APPLICATION OF NEW GENOMIC METHODS TO THE CHARACTERIZATION OF *ARABIDOPSIS THALIANA* PHOTOMORPHOGENESIS

A Dissertation

by

ROBERT WAYNE CORBETT

Submitted to the Office of Graduate Studies of
Texas A&M University
in partial fulfillment of the requirements for the degree of

DOCTOR OF PHILOSOPHY

August 2005

Major Subject: Plant Physiology

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Approved by:

Chair of Committee, Alan Pepper
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Carol Loopstra
James Manhart
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ABSTRACT

Application of New Genomic Methods to the Characterization of *Arabidopsis thaliana* Photomorphogenesis. (August 2005)

Robert Wayne Corbett, B.S. Texas A&M University

Chair of Advisory Committee: Dr. Alan Pepper

The ability of plants to not only detect but also adjust to their environment is crucial for their survival. The genes involved in photomorphogenesis – developmental changes in response to light – and their regulation have long been of interest to researchers. While the phytochrome and cryptochrome photoreceptors have been isolated and partially characterized, the downstream components of the light signaling pathway which transmit the perceived light signals and regulate gene expression are still being discovered. A negative regulator of photomorphogenesis, *DET1* (*de-etiolated 1*), was discovered in a mutant screen for plants that develop a light grown phenotype in the dark. *DET1* is nuclear localized, but its exact function remains unknown. Two contrasting mechanisms for the role of DET1 in the regulation of gene expression have been proposed based on studies of the tomato and human orthologs of DET1. In order to reveal the mechanism and molecular context of DET1 action, suppressor mutant screens were employed to discover additional genes acting in conjunction with *DET1* (designated as *TED* genes). In this research, new genomic methods were developed and

employed to identify the genes underlying the *ted1-1SD* and *ted2-1D* suppressor mutations.

A long hypocotyl QTL and suppression of the *det1-1* dark grown phenotype by the Bensheim (Be-0) ecotype of Arabidopsis mapped to the *HAT4* gene, a homeobox-domain leucine-zipper transcription factor involved in shade-avoidance responses. Sequence analysis uncovered two functionally distinct alleles of *HAT4* in the Be-0 alleles of *HAT4* compared to the genomic standard Columbia (Col-0) ecotype. Expression analysis showed that in addition to negative autoregulation by itself, *HAT4* is also negatively regulated by *DET1.* The *ted2-1D* mutation was mapped to a 57 Kbp interval on chromosome I containing three likely candidate genes. Suppression of the *det1-1* phenotype by *ted2-1D* is overdominant which is highly unusual and typically associated with hybrid vigor or heterosis traits. The discovery of the genes underlying the *ted1-1SD* and *ted2-1D* suppressor mutations have furthered the understanding of the role for *DET1* in regulation of photomorphogenesis as well as mechanisms involved in overall gene regulation during light signaling.

I dedicate this dissertation to my family and friends

who never gave up on me during the rough times and were there

with me to celebrate the good times of graduate school.

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CHAPTER I

INTRODUCTION

The ability of plants to adapt to their surrounding environment is what has allowed them to spread throughout the planet and inhabit many different environmental niches. Unlike animals, plants are sessile in nature and do not have the ability to move to a more favorable environment. Sunlight is one of the most important environmental influences on plant growth. Not only does sunlight provide the plant the energy it needs in order to perform photosynthesis, but it is also an environmental signal affecting many aspects of plant growth and development from germination to senescence. The amount and quality of light a plant receives have dramatic effects on many aspects of plant growth and development. While some of the major genes involved in perceiving light and controlling plant responses have been identified, there are still many others that play key rolls in the intricate pathways involved in light responses. Further understanding of these genes and their function in the plant responses to light conditions in the environment will allow a greater understanding of how plants have been able to adapt to environmental conditions that are less than favorable to survive and reproduce.

Plants have evolved an exquisite sensory system for monitoring light in their environment. Intensity, quality, direction and duration of light are continuously

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This dissertation follows the style and format of *The Plant Cell.*

monitored and the information is used to control all aspects of plant growth and development (Whitelam *et al*., 1998). Perception of the different types of light involve the phytochromes which perceive red and far-red light, cryptochromes and phototropins which perceive blue light, and a yet unidentified receptor for UV-B light (Chen *et al*., 2004). While the functions of these photoreceptors are known, the downstream components of the light signaling pathway have yet to be completely unraveled.

Photomorphogenesis is loosely defined as the diverse set of developmental responses to light (Furuya, 1987; Pepper *et al*., 1993). Of particular interest to this research is the process of seedling de-etiolation from the dark (skotomorphogenic) form to the light (photomorphogenic) form. The de-etiolation process is phenotypically marked by the opening of the cotyledons (seed leaves), initiation of leaf development by the apical meristem, and the decrease in the rate of hypocotyl elongation (Pepper *et al*., 1993). Etiolation is thought to be an environmental adaptation to low levels of light such as in the case of germination deep within the soil or shade (Hart, 1988). Etiolation allows the plant to forgo leaf development and use its energy stores for elongation of the hypocotyl or stem in order to reach better light conditions.

In order to study the genetic regulation of the de-etiolation process, mutational analysis was used to find mutants that did not follow the normal photomorphogenetic pattern. One family of mutations, designated *det* for *de-etiolated,* showed a de-etiolated phenotype (i.e. their cotyledons expanded, true leaves were initiated, and hypocotyl elongation was arrested) when the plants were grown in complete darkness or at low levels of light where they should have shown etiolated and shade avoidance phenotypes,

respectively (Chory *et al*., 1989b). These mutants include *det1* (*de-etiolated 1*), *cop1* (*constitutively photomorphogenic 1*), *cop9*, *det2* and *fus6* (*fusca 6*, "little red plant") (Mayer *et al*. 1996). Mutations in this family are recessive, suggesting that they are involved in the negative regulation of de-etiolation (Pepper *et al*., 1994). Another family of mutations, designated *hy* for *long hypocotyl*, showed a partially etiolated phenotype (i.e. their hypocotyls continued to elongate) when the plants were grown under normal light conditions. While most of the *hy* mutations affect pathways in the synthesis of phytochromes and cryptochromes, the *hy5* mutation is located in a gene encoding a basic leucine zipper (bZIP) transcription factor and is a positive regulator of photomorphogenesis.

The *det1* mutation is one of the best studied members of this family of mutations. It is pleiotropic, affecting many developmental processes. DET1 is a novel, nuclear localized protein that is a negative regulator of light-mediated transcription (Pepper *et al*., 1994). However, no DNA binding activity was detected. DET1 may act via protein-protein interactions, but this hypothesis has not been thoroughly tested.

DET1 may also be involved in pathways other than the light signal transduction pathway. Mayer *et al*. (1996) demonstrated that genes typically associated with defense and stress responses such as phenylalanine ammonia-lyase (PAL), glutathione S-transferase (GST), and pathogenesis related (PR) genes (PR-4), are activated in *det1* mutants in the absence of pathogen infection (Mayer *et al*., 1996). They also demonstrated inappropriate tissue specificity of several of these genes. Because of these examples, DET1 was hypothesized to be a negative regulator of signal transduction

pathways other than light. However, an important control experiment, the effect of light on the expression of these stress and pathogen response genes, was not performed in this study.

In addition to *DET1*, additional possible regulators of photomorphogenesis have been examined. The *COP/FUS* genes have similar mutant phenotypes to *DET1* and are also considered to be master regulators of light responses. Multiple transcription factor genes have also been shown to be targets of phytochrome and cryptochrome signaling including the LONG HYPOCOTYL 5 (*HY5*), LATE ELONGATED HYPOCOTYL (*LHY*), and *HAT4* which is involved in the shade-avoidance response (Tepperman *et al*., 2001). As with the *DET/COP/FUS* regulators, these genes have an effect on hypocotyl length which is the most commonly used quantitative trait to measure photomorphogenetic responses in plants.

Currently there are two conflicting models of the molecular function of *DET1* based on biochemical experiments in tomato and in humans. In tomato, DET1 binds to the nonacetylated amino-terminal tails of histone H2B (Benvenuto *et al*., 2002). In this model, binding to histone H2B prevents acetylation and causes the chromatin to remain in the condensed state, thus preventing expression of genes located within the condensed chromatin. This activity negatively regulates the expression of genes required for de-etiolation. In an alternative model of *DET1* function, derived from studies of the human ortholog, DET1 is involved in the ubiquitin-mediated degradation of transcription factors via the ubiquitin-proteosome system (Wertz *et al*., 2004). Additional support for this second model has been provided by studies in *Arabidopsis* showing that DET1 forms a

complex with two additional proteins, constitutive photomorphogenic 1 (COP1) and damaged DNA-binding protein 1 (DDB1), and that this complex targets transcription factors, such as *HY5*, for degradation by ubiquitin dependant pathways (Yanagawa *et al*., 2004).

In an attempt to further dissect the molecular context of *DET1* action, a genetic approach was used: screening for extragenic suppressors of the *det1-1* mutant with the goal of discovering genes that may act upstream or downstream of *DET1* or circumvent *DET1* altogether (Pepper and Chory, 1997). Of the six suppressor loci isolated (designated as *ted* for the reversal of *det1*), two were in previously isolated genes and a third has been characterized by further studies. The suppressor loci *ted4* and *ted5* are allelic to the previously described loci *HY5* (long hypocotyl 5) and *HY1* (long hypocotyl 1) respectively. *HY5* encodes a basic leucine zipper (bZIP) transcription factor that is involved in the positive regulation of photomorphogenesis (Ang *et al*., 1998; Chattopadhyay *et al*., 1998) and is targeted for proteosome-mediated degradation in the dark by the negative regulator *COP1* (a ubiquitin ligase) (Osterlund *et al*., 2000; Saijo *et al*., 2003). *HY1* encodes a heme oxygenase responsible for catalyzing the reaction that generates the precursor for the tetrapyrrole chromophore subunit of the phytochrome chromoprotein (Davis *et al*., 1999; Muramoto *et al*., 1999). The *ted3* locus encodes a protein with a C_3HC_4-type RING finger motif and two putative-membrane spanning domains that is homologous (~20 to 24% identity) to the human and yeast *PEX2* genes involved in peroxisome assembly and matrix protein import (Hu *et al*., 2002). Although *ted3* suppresses the dark-grown phenotype of *det1-1*, the exact function of the

peroxisome in regulation of photomorphogenesis is still unclear.

The following work examines both the natural variation in photomorphogenesis seen in different ecotypes of *Arabidopsis*, along with the *ted1-1SD* and *ted2-1D* extragenic suppressors of *det1-1*, in an attempt to further understand the role of *DET1* in the control of photomorphogenesis in plants. The genetic loci examined in this project, including *ted1-1SD* and *ted2-1D*, behaved essentially as QTL (quantitative trait loci) rather than as simple Mendelian loci. Identifying these loci at the molecular level required characterization of large scale mapping populations. To aid in the analysis of these large populations, new large-scale genomic methods were developed and employed.

CHAPTER II

NATURAL VARIATION IN *ARABIDOPSIS* SEEDLING PHOTOMORPHOGENESIS[†]

OVERVIEW

Natural genetic variation present among accessions of *Arabidopsis thaliana* (L.) Heynh. (commonly referred to as 'ecotypes') is a valuable, yet under-exploited genetic resource for the study of plant development, physiological, and evolutionary responses to the environment. Seedling photomorphogenic responses were surveyed in a set of 11 *Arabidopsis thaliana* accessions collected from a variety of edaphic habitats and geographic locations. We observed substantial variation in light-dependent hypocotyl growth responses in a variety of light conditions (white, red, blue, far-red enriched light). The genetic basis for differences in hypocotyl growth responses to light between the Columbia (Col-0) and (Be-0) accession was examined in an F_2 population. Quantitative genetic and quantitative trait locus (QTL) analyses were consistent with a model in which differences in light responses were conditioned by a single major gene with semi-dominant effect, located on chromosome 4. Further experiments suggested that the genetic difference governing hypocotyl variation in this cross might be allelic to *ted1*, an

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[†] Reprinted with permission from "Natural variation in *Arabidopsis* seedling photomorphogenesis reveals a likely role for *TED1* in phytochrome signaling" by A.E. Pepper, R. W. Corbett & N. Kang, 2002, *Plant, Cell and Environment*, 25, 591-600. Copyright 2002 by Blackwell Publishing Ltd.

R.W. Corbett's contribution to this publication included SSLP and CAPS molecular marker development and genotyping in the Be-0 × Col-0 population, for QTL analysis used in mapping of the putative *TED1* allele.

extragenic suppressor of the *de-etiolated* mutant *det1*, which was identified as an ethyl-methane sulphonate-induced mutation. This finding supports a role for *ted1* in photomorphogenic signaling.

INTRODUCTION

The genetic control of photomorphogenesis in *Arabidopsis thaliana* (L.) Heynh. is being intensively studied using induced mutations. This work has led to the functional characterization of several of the primary photoreceptors including *PHYA* (Dehesh *et al*., 1993; Nagatani *et al*., 1993; Parks and Quail, 1993; Whitelam *et al*., 1993), *PHYB* (Reed *et al*., 1993; Somers *et al*., 1993), and *CRY1* (Ahmad and Cashmore, 1993), as well as a host of downstream signaling molecules (Deng and Quail, 1999; Neff *et al*., 2000; Smith, 2000). Plant photomorphogenesis has recently emerged as a general model for the interactions of environment with development (Schmitt and Wulff, 1993; Schmitt *et al*., 1995; Smith, 1995; Ballare and Scopel, 1997; Callahan *et al*., 1997; Pigliucci, 1998; Pigliucci and Schmitt, 1999; Schmitt *et al*., 1999; Smith, 2000).

The examination of natural genetic variation present in Arabidopsis accessions (commonly referred to as 'ecotypes') has proven to be an important tool for the discovery of new genes affecting a given biological process, as well as the isolation of novel alleles at loci that have been previously discovered through induced mutation (Alonso-Blanco and Koornneef, 2000). Studies of natural genetic variation have been critical to our understanding of the genetic regulation of flowering time (Lee *et al*., 1993; Clarke and Dean, 1994; Kowalski *et al*., 1994; Mitchell-Olds, 1996; Alonso-Blanco *et*

al., 1998), and have led to important discoveries in such diverse fields as plant defenses against pathogens (Kunkel *et al*., 1993; Mahajan *et al*., 1998) and the circadian clock (Swarup *et al*., 1999).

Natural genetic variation among Arabidopsis accessions appears to be an important new asset for the study of seedling photomorphogenetic responses (Maloof *et al*., 2000). For example, an examination of natural variation present between Columbia (Col-0) and Landsberg-*erecta* (La-*er*) by Yanofsky *et al*. (1997) identified two novel loci involved in perception of the very-low-fluence (VLF) light by Phytochrome A. Aukerman *et al*. (1997) defined the phenotype of a *phyD* apoprotein null mutation through the discovery of a natural loss-of-function (14 bp deletion) allele present in the Wassilewskija (Ws-0) accession.

In order to more fully explore the natural genetic variation in photomorpho-genetic responses, we examined natural variation in seedling de-etiolation under a variety of light conditions in a collection of 11 single-seed descent populations. These populations were derived from lines that were originally collected from a variety of geographic locations and edaphic environments. We used hypocotyl length as a convenient and quantitative measure of de-etiolation responses. In order to uncover the genetic basis of intraspecific variation in hypocotyl responses, we employed inter-accession crosses, followed by quantitative trait loci (QTL) analyses. The framework molecular marker set used in QTL analysis included cleaved amplified polymorphic sequence (CAPS) and simple sequence length polymorphism (SSLP) markers derived from genes previously determined, through induced mutation studies, to have roles in

seedling photomorphogenesis such as *PHYA*, *PHYB, HY5* (Oyama *et al*., 1997), *DET1* (Chory *et al*., 1989a; Pepper *et al*., 1994), *CCA1* (Wang and Tobin, 1998), *COP1* (Deng *et al*., 1991; Deng *et al*., 1992), and *COP9* (Wei *et al*., 1994), as well as other CAPS and SSLP markers closely linked to additional genes known to encode photomorphogenetic regulators including *PHYD*, *PHYE* (Clack *et al*., 1994), *CRY1* (Ahmad and Cashmore, 1993), *CRY2* (Lin *et al*., 1998) and others. With this set of markers, linkage to one of these 'candidate genes' could, if present, be readily detected.

MATERIALS AND METHODS

Plant Materials

Seed stocks used in this study are listed in Table 2.1. With the exception of Col-0, these were obtained from the Arabidopsis Biological Resource Center (ABRC) at Ohio State University. These stocks were originally collected in a variety of geographic locations and edaphic habitats. Col-0 seeds were obtained from the laboratory stocks of Joanne Chory. Single-seed descent populations were established by harvesting seeds from individual plants. Seeds for phenotypic analyses were harvested from plants grown in a temperature controlled growth room (24°± 1° C) with 16 h of white light (100 μmol m^{-2} s^{-1}) per day. Seeds were collected when the majority of silique were yellow/brown and dehiscent. After collection, seeds were dried at room temperature in low-humidity conditions (< 40% relative humidity) for a minimum of 21 days prior to germination.

Table 2.1. Plant materials used in this study. With the exception of Col-0, all accessions were obtained from the Arabidopsis Biological Resource Center (ABRC). ABRC stock number is indicated.

Accession	Source (ABRC no.)	Origin and habitat
Ag-0	ABRC (CS901)	Argentat, France. Railway ballast.
Be-0	ABRC (CS6613)	Bensheim, Germany.
Bch-3	ABRC (CS958)	Buchen, Germany. Deposited sand.
Col-0	J. Chory	Landsberg, Germany (Redei, 1993).
El-0	ABRC (CS1134)	Ellershausen, Germany, Limestone.
En-1	ABRC (CS1136)	Enkheim, Germany, Sandy loam.
Est-0	ABRC (CS911)	Estland, Russia.
Je-0	ABRC (CS1246)	Jena, Germany. Shaded new sandstone.
Kil-0	ABRC (CS1270)	Kilean, UK. Mica schist.
Lc-0	ABRC (CS1306)	Loch Ness, UK. Moine schist.
No-0	ABRC (CS3081)	Nossen, Germany.

Light Sources

White light was supplied by an equal mixture of cool white (CW) and Grow-lux wide-spectrum (WS) fluorescent bulbs (Sylvania, Danvers, MA, USA). Red light was supplied by CW fluorescent bulbs filtered through a Kopp 2-73 red glass filter (Kopp Glass, Pittsburgh, PA, USA). Blue light was supplied by CW fluorescent bulbs filtered through a Kopp 5-57 blue filter. Far-red enriched light was provided by a 60W incandescent bulb filtered through a Kopp 2-64 glass filter (R/FR ratio of ±0.32). Dark experiments were performed in a passively-ventilated dark box. Fluence rates of white, red, blue, yellow and green light were measured with a quantum photometer (model LI-189, Li-Cor, Lincoln, NE, USA). Fluence rates of far-red light were measured using a radiometer (model IL1400, International Light, Newburyport, MA, USA) with FR probe (model SEL033, International Light).

Hypocotyl Measurements

Seeds were surface sterilized (Chory *et al*., 1989b), resuspended in sterile 0.1% phytagar, then cold treated for 84 hours at 4° C. Plates for phenotypic analyses were prepared by aliquotting 35 ml of MS/phytagar/2% (w/v) sucrose media into 25 mm x 100 mm polystyrene petri dishes. After cooling, plates were allowed to dry for 48 hours prior to use. Seeds were dispersed onto solid agar media in a 7 mm grid pattern to ensure even spacing. Each plate was divided into six sectors, and 30 seeds of each population were distributed into each sector. To ensure optimal germination seeds were illuminated for 4 hours with white light (100 μmol m^{-2} s^{-1}) prior to various light treatments. Seedlings

were grown for 8 days in the light conditions indicated prior to hypocotyl measurement. Hypocotyls were straightened using forceps if necessary, then measured under a stereo dissecting-microscope using a 0.5 mm scale ruler. Hypocotyls of seedlings that were appressed to the agar media, as well as those with obvious developmental abnormalities, were not measured.

Genetic and Molecular Marker Analysis

The Arabidopsis genetic methods employed were described previously (Chory *et al*., 1989b). Genomic DNAs were isolated using the micropreparation method described by Pepper and Chory (1997). Cleaved amplified polymorphic sequence (CAPS) and simple sequence length polymorphism (SSLP) marker analyses were performed using standard conditions (Konieczny and Ausubel, 1993; Bell and Ecker, 1994). Primer sequences for the majority of these markers have been published (Konieczny and Ausubel, 1993; Bell and Ecker, 1994; Lukowitz *et al*., 2000) or are available over the world-wide web at The Arabidopsis Information Resource (TAIR) web site (www.arabidopsis.org). Polymerase chain reaction (PCR) based markers for *HY5* (chromosome 5), and *COP9* (chromosome 4) and the bacterial artificial chromosome T6K21 (chromosome 4) were developed in our laboratory. Primer information on these markers, as well as profiles of all markers in the Bensheim (Be-0) genetic background will be submitted to The Arabidopsis Information Resource (TAIR) for inclusion in developing molecular marker databases. Genotypes of inter-accession F_1 hybrids were confirmed by diagnostic SSLP fingerprinting using markers nga8 (chromosome 4) and

nga106 (chromosome 5).

Analysis of SSLP amplification products was performed using 2% standard agarose plus 2% Metaphor® agarose (Cambrex, North Brunswick, NJ, USA) gels. Samples were electrophoresed at 5.3V/cm in 0.5X TBE buffer (45 mM Tris-Borate, 1 mM EDTA, pH 8), with buffer-chilling to 4°C. When high resolution was required, we employed a vertical acrylamide gel electrophoresis system in which samples were electrophoresed at 12V/cm in a 10 cm high x 33 cm wide x 1 mm thick vertical gel rig (CBS Scientific, Del Mar, CA, USA) containing 6% polyacrylamide with 10% v/v Spreadex NAB polymer® (Elchrom Scientific, Cham, Switzerland) in 1X TAE buffer (45 mM Tris-Acetate, 1 mM EDTA, pH 8). QTL analysis was performed using the composite interval mapping (CIM) method (Jansen, 1993; Zeng, 1993, 1994) of QTL Cartographer ver. 1.21 (Basten *et al*., 1994; Basten *et al*., 2001). Seedlings homozygous for the *det1-1* and *det1-4* mutations were identified by PCR-based diagnostic methods as described previously (Pepper and Chory, 1997).

RESULTS

Genetic and Environmental Components of Variation

Single-seed descent populations were obtained from a set if 11 Arabidopsis accessions originally collected from a variety of edaphic habitats and geographic locations (Table 2.1). Seedling hypocotyl lengths from each population were measured after growth in complete darkness, low white light (15 μmol m^{-2} s^{-1}), moderate white light (45 μmol m^{-2} s^{-1}), blue (15 μmol m^{-2} s^{-1}), red (23 μmol m^{-2} s^{-1}), and far-red enriched light (12 μmol m^{-2} s^{-1}). For all light conditions, each population had an approximately normal distribution. These hypocotyl measurements are summarized in Figures 2.1A and 2.1B.

Since the Col-0 line was derived from laboratory stocks that had been self-propagated for decades prior to the start of this experiment (Redei, 1993), we assumed this population to be monomorphic at all loci affecting hypocotyl length (i.e. the genetic component of variance, $V_G = 0$ for Col-0). Since the seedlings were grown evenly spaced on sterile media containing supra-optimal levels of nutrients, the environments experienced by each seedling were largely similar, if not identical. Therefore, we assumed the environmental component of variance, $V_E \pm 0$ for all populations. The observed phenotypic variance for each population in each light condition, which should be $\pm V_G$, is listed in Table 2.2. Lc-0 had significantly higher phenotypic variance than Col-0 in all light conditions tested. Similarly, En-1 had a significantly higher phenotypic variance than Col-0 in the low white light experiment, and both En-1 and Jena (Je-0) had a significantly higher variance than Col-0 in blue light. These findings suggested that for

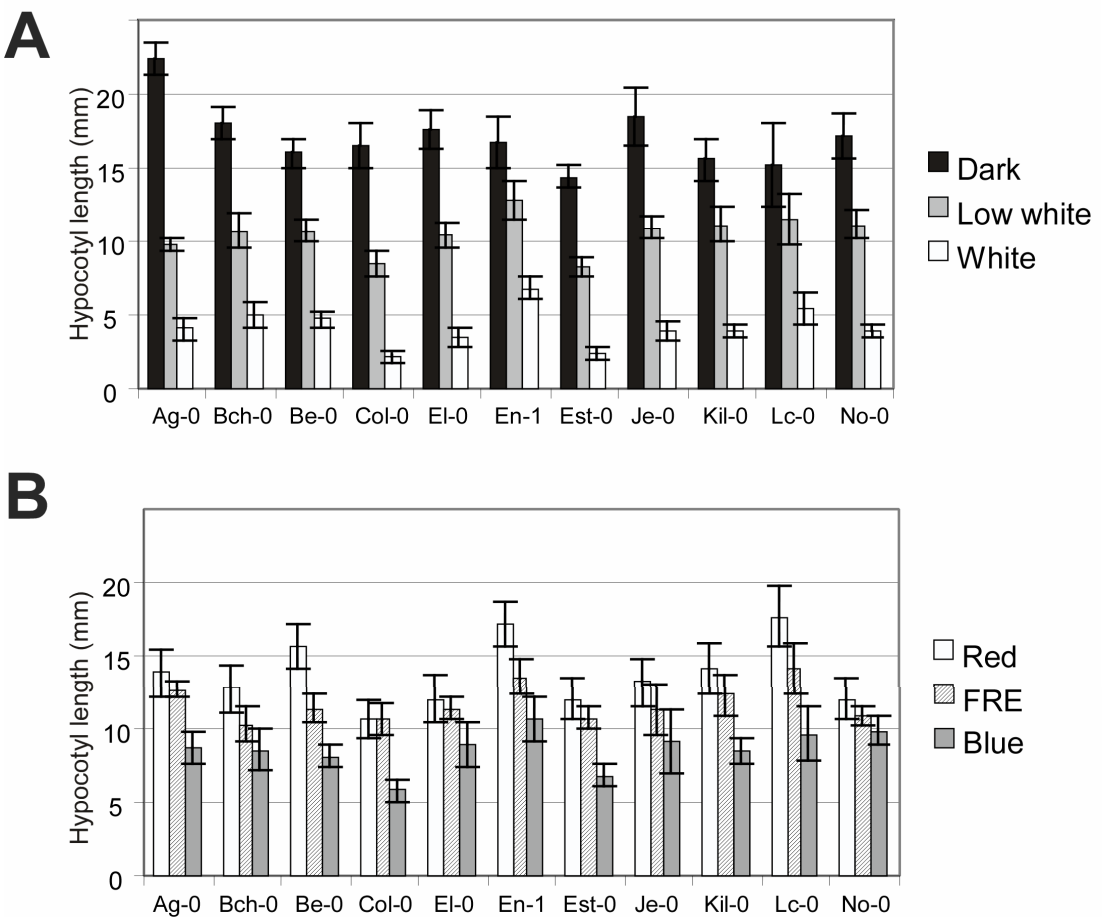


Figure 2.1. Natural variation in hypocotyl growth responses among accessions of *A. thaliana.*

(A) Hypocotyl length in darkness, low white light (15 μmol m^{-2} s^{-1}) and moderate white light (45 μmol m^{-2} s^{-1}) conditions.
(B) Hypocotyl length in red light (23 μmol m^{-2} s^{-1}), far-red enriched (FRE) light (12 μmol m^{-2} s^{-1}), and blue light (15 μmol m^{-2} s^{-1}). Error bars indicate standard deviations.

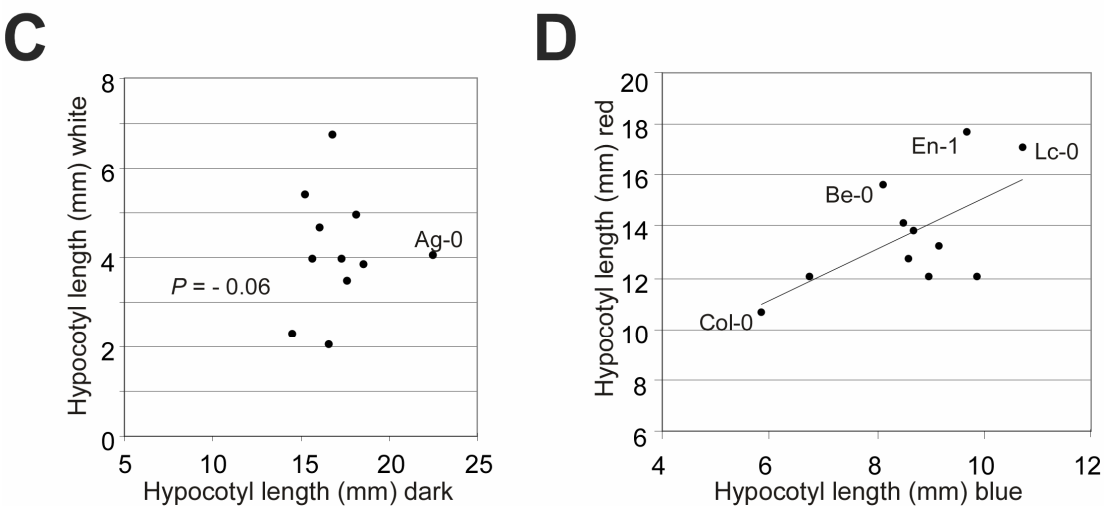


Figure 2.1. Continued.

(C) Hypocotyl length in darkness vs. moderate white light.
(D) Hypocotyl length in red vs. blue light. Accessions with notable phenotypes (Ag-0, Col-0, Be-0, En-1, Lc-0) are indicated.

Table 2.2. Variances and relative plasticity indices (RPI[1]) for single-seed descent populations in the light conditions indicated.

	Ag-0	Bch-3	Be-0	Col-0	El-0	En-1	Est-0	Je-0	Kil-0	Lc-0	No-0
RPI_{white}	5.5	3.62	3.43	8	4.99	2.47	6.13	4.76	3.9	2.79	4.3
RPI_{red}	1.63	1.42	1.03	1.56	1.46	0.98	1.2	1.4	1.1	0.87	1.43
RPI_{fre}	1.78	1.76	1.41	1.57	1.54	1.25	1.35	1.64	1.27	1.08	1.58
RPI_{blue}	2.58	2.11	1.98	2.84	1.96	1.57	2.14	2.02	1.84	1.58	1.75

[1]RPI (relative plasticity index) is the ratio of hypocotyl length in the dark to hypocotyl length in the indicated light condition.

the Lc-0 population, and possibly the En-1 and Je-0 populations, V_G may be greater than 0. Therefore these particular populations may not be monomorphic at all loci effecting hypocotyl length. For all other populations, we could not exclude the hypothesis that these were indeed monomorphic at all loci affecting hypocotyl length.

Most of the Natural Variation in Hypocotyl Length is Light-dependent

Hypocotyl growth is determined by a number of extrinsic and intrinsic factors in addition to light. These include temperature, humidity, nutrient availability, mechanical stress, seed size and phytohormone activity (Zacarias and Reid, 1992; Qiu and Mosjidis, 1993; Sawan *et al*., 1993; Wang *et al*., 1993; Gray *et al*., 1998; Hong and Vierling, 2000; van der Weele *et al*., 2000; Weinig, 2000a). Several of these factors interact with light signals to regulate hypocotyl growth responses. If phenotypic variation among natural populations is the result of genetic factors that affect hypocotyl growth in a manner that is completely independent of light (such as factors effecting seed size or maximal intrinsic growth rate), we would expect this variation to be evident in dark-grown, as well as light-grown seedlings. Analysis of variance (ANOVA) did reveal statistically significant variation in the means of dark-grown hypocotyl length among the populations ($P = 3.1 \times e^{-23}$). However, with the exception of the significant outlier Argentat (Ag-0), the means of the dark-grown hypocotyl lengths of many of the study populations were quite similar, and in some cases statistically indistinguishable (Figure 2.1A). ANOVA analysis of the hypocotyl length data set without Ag-0 revealed far less significant variation ($P = 3.28 \times e^{-15}$).

In all light conditions, however, there was remarkable variation for hypocotyl length among our study populations (Figures 2.1A and 2.1B). For example, ANOVA of white light grown samples showed highly significant 'between groups' variance ($P = 3.1 \times e^{-88}$). In this light condition, Enkheim (En-1) had the longest mean hypocotyl length (6.8 ± 0.7 mm), while Col-0 had the shortest (2.1 ± 0.3 mm). Within each population, the mean hypocotyl length of dark-grown seedlings was not correlated with the mean length of white light grown seedlings (Figure 2.1C), indicating that most of the observed variation is light-dependent.

In order to quantify phenotypic plasticity in response to light, we used a ratio of hypocotyl length in dark to hypocotyl length in a given light condition. Here, we refer to this ratio as the relative plasticity index (RPI). For example, the ratio of dark-grown to white light-grown hypocotyl lengths, designated RPI_{white}, ranged from a high of 8.0 in Col-0 to a low of 2.47 in En-1 (Table 2.2). This remarkable 3.2 fold range of RPI_{white} values demonstrates that there is substantial variation in phenotypic plasticity responses among natural populations of Arabidopsis. Substantial variations in the RPIs are also seen in blue, red and far-red enriched light (Table 2.2). These findings demonstrated that the major component of variation in hypocotyl length among the populations tested is strongly light-dependent, indicating that there is substantial genetic variation in 1) light signaling pathways, or 2) other developmental or growth-related pathways that have strong interactions with light signals.

Natural Variation in Hypocotyl Responses to Blue and Red Light

Seedling hypocotyl responses to red light are largely mediated by the PHYB photoreceptor (Reed *et al*., 1993; Reed *et al*., 1994; Casal and Mazzella, 1998) with PHYD and PHYE having minor roles (Aukerman *et al*., 1997; Devlin *et al*., 1998; Devlin *et al*., 1999). Responses to blue light are mediated largely by CRY1, with PHYA and CRY2 playing subsidiary roles (Ahmad and Cashmore, 1993; Casal and Mazzella, 1998; Lin *et al*., 1998; Neff and Chory, 1998). To determine if natural variation in seedling hypocotyl responses was dependent on spectral conditions, we examined our populations in narrow spectrum red light, blue light and in a far-red enriched light condition (R/FR ratio of approximately 0.32) similar to the light environment under a dense canopy (Figure 2.1B). Based on RPI (Table 2.2), the Col-0 ecotype was the most developmentally responsive to blue light, while Lc-0 was least responsive. En-1, Lc-0 and Bensheim (Be-0) were the least responsive to red light. When hypocotyl lengths in red light were plotted against those in blue (Figure 2.1D), nearly all of the ecotypes fell into a loose quasi-linear cluster, with Col-0 as the accession with the shortest hypocotyl in both red and blue light. In none of the accessions was variation in hypocotyl length (with respect to Col-0) exclusively dependent on either red or blue light. In no case could the observed variation be ascribed to a simple loss of function allele in one of the major photoreceptors (*PHYA*, *PHYB*, *CRY1*).

The genetic bases for differences in light responses among the extreme populations was examined through crosses between the well-characterized Col-0 accession and the less well characterized Be-0, En-0 and Lc-0 accessions. Because of the

likelihood of intra-population genetic variation within Lc-0 and En-0, these lines were further self-propagated for several generations prior to outcrossing to Col-0. The results of these latter two inter-accession crosses will be presented elsewhere.

Hypocotyl Length in the F_2 Generation of a Col-0 × Be-0 Cross is Consistent with Action of a Single Major Gene

Hypocotyl phenotypes of F_1 and F_2 generations of a Col-0 × Be-0 cross were determined in a moderate white light condition (40 μmol m^{-2} s^{-1}), that gave excellent discrimination of the Col-0 and Be-0 parental phenotypes (Figure 2.2). In this analysis, F_1 individuals had hypocotyl lengths that were intermediate between those of the Col-0 and Be-0 parents. F_2 individuals showed a semi-continuous distribution of phenotypes with a minor peak at 2.5 mm, and more distinct peaks at 3.5 mm and 5.0 mm. These peaks roughly corresponded to the population means of the Col-0 parent, the F_1 individuals, and the Be-0 parent, respectively. By equally partitioning the individuals falling into the minor 'troughs' to adjacent peaks, we estimated that the numbers of individuals falling into the three peaks to be 19, 37, and 16, respectively. This is a close fit to a 1:2:1 distribution ($\chi^2 = 0.19$, $P > 0.90$). From these data, a simple model emerged in which variation in white light-grown hypocotyl length between Be-0 and Col-0 was conditioned, at least in part, by the action of a single major gene with semi-dominant effects. A single major-gene model was supported by the apparent absence of individuals with obvious transgressive phenotypes in the F_2 population (which may result from the additive action of combinations of alleles at multiple loci).

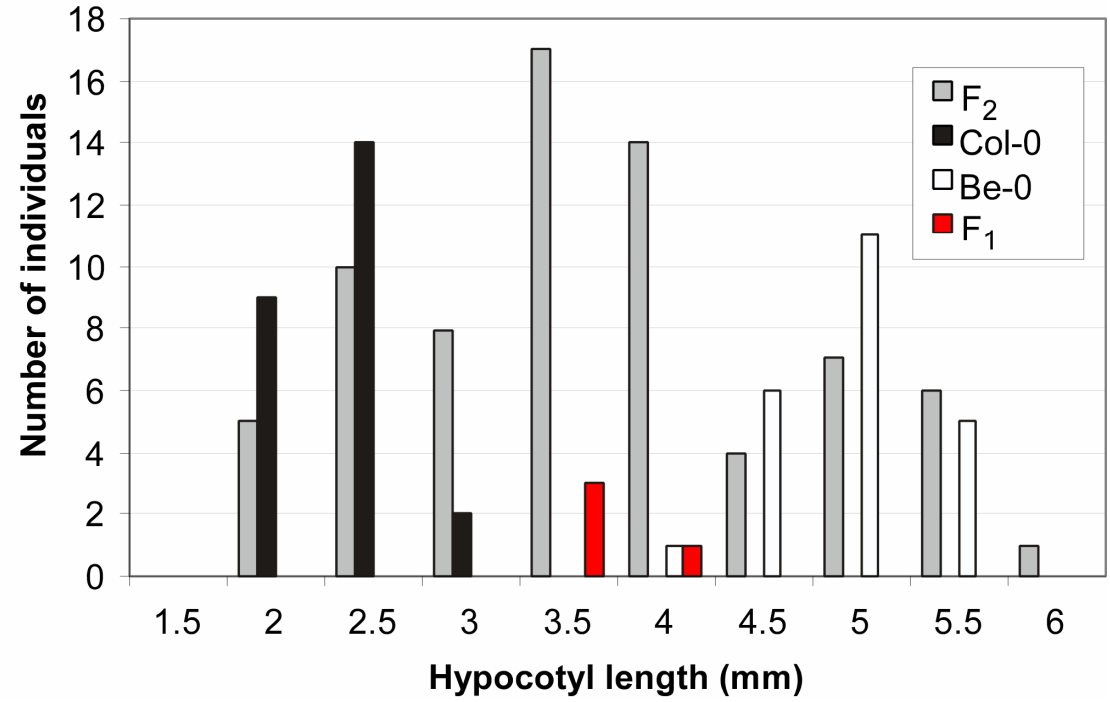


Figure 2.2. Hypocotyl length distribution in an F_2 population from a Col-0 × Be-0 cross.

Histogram showing the 72 individuals derived from a Col-0 x Be-0 cross (light grey). Seedlings were grown in white light (45 μmol m^{-2} s^{-1}) for 8 days. Phenotypic distributions of the Col-0 (black) and Be-0 (white) parents, as well as a small set of F_1 hybrids are also indicated (dark grey).

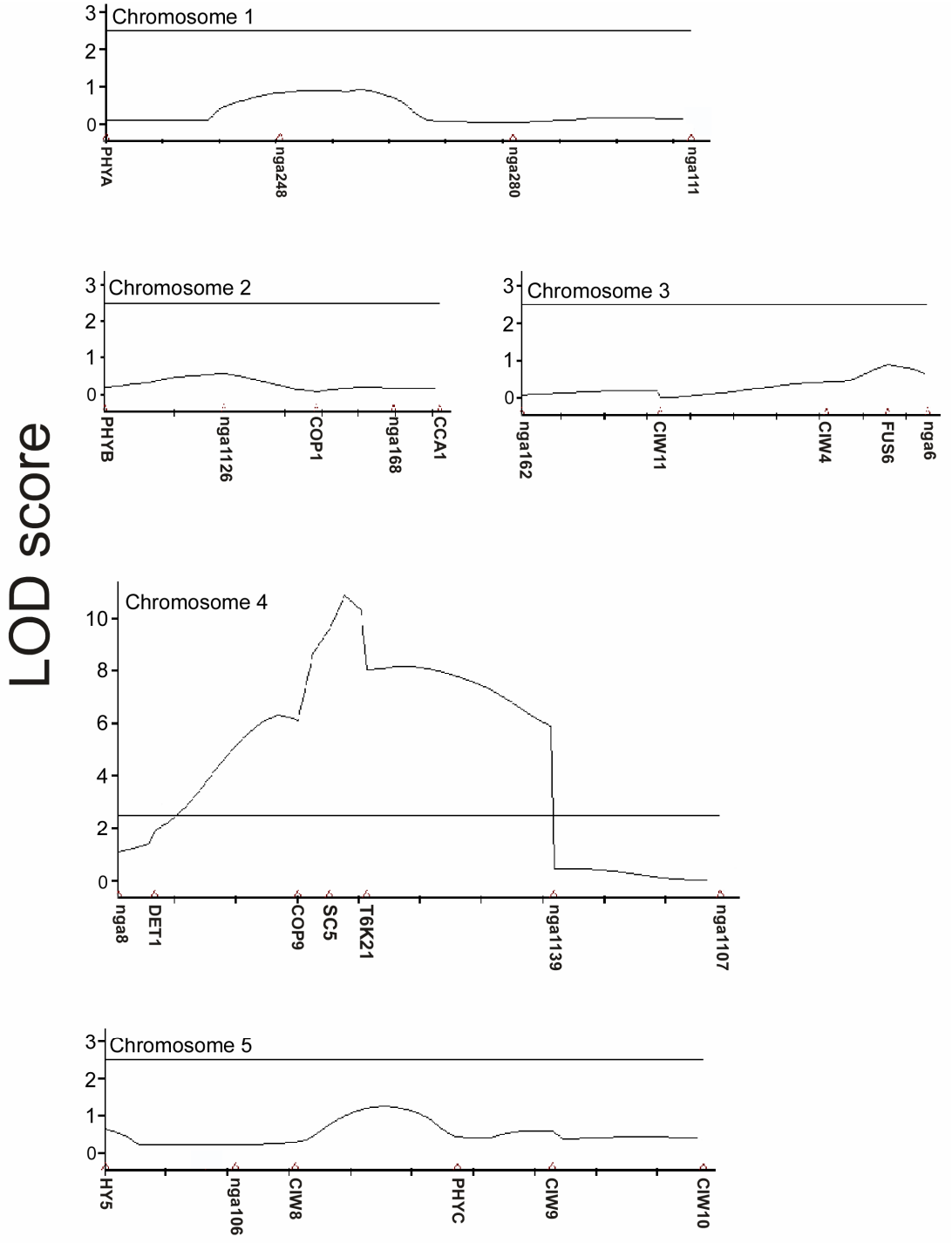
QTL Analysis

To identify the specific chromosomal regions associated with natural variation in hypocotyl responses in the Be-0 × Col-0 F_2 population, genomic DNAs were isolated from 48 F_2 individuals for quantitative trait locus (QTL) analysis (Lander and Botstein, 1989). Progeny were examined with a framework set of 28 PCR-based markers with an average interval of 17 cM. Composite interval mapping (CIM) identified a strong QTL within a broad region of chromosome 4 (Figure 2.3). The interval with the greatest likelihood of containing a significant QTL, with a logarithm-of-odds (LOD) score of greater than 10.7, was between the CAPS marker SC5 and an SSLP marker derived from the BAC T6K21. Candidate photomorphogenetic regulatory genes in this region of the genome include *PHYD, PHYE* (Clack *et al*., 1994), and *TED1*(Pepper and Chory, 1997) an as yet uncloned gene identified by extragenic suppressors of the *de-etiolated det1* mutant, which were generated by ethlylmethane sulfonate (EMS) mutagenesis of *det1-1* (Col-0 genetic background). Other photomorphogenetic loci that map within the broadest interval identified by the CIM experiment were *COP9* (Wei *et al*., 1994) and *RED1* (Wagner *et al*., 1997), which are at the margins of the interval.

Loss of function mutations in both *PHYD* and *PHYE* were reported to have subtle, if at all detectable, effects on hypocotyl length in white light (Aukerman *et al*., 1997; Devlin *et al*., 1998; Devlin *et al*., 1999). In contrast, a line carrying the mutant allele *ted1-1SD*, was observed to give rise to progeny with a moderate long hypocotyl phenotype in white light when outcrossed to wild type Col-0 (Pepper and Chory, 1997). This allele partially suppressed the *det1* phenotype in a semi-dominant manner, and was

Figure 2.3. QTL regulating hypocotyl length.

Composite interval mapping (CIM) of QTL regulating hypocotyl length (in white light) in a Be-0 x Col-0 population. The framework set of marker loci are indicated. The horizontal line indicates a LOD = 2.5 significance threshold (Lander & Botstein 1989).


LOD score
Chromosome 1
3
2
1
0
PHYA
nga248
nga280
nga111
Chromosome 2
PHYB
nga1126
COP1
nga168
CCA1
Chromosome 3
nga162
CIW11
CIW4
FUS6
nga6
Chromosome 4
10
8
6
4
2
0
nga8
DET1
COP9
SC5
T6K21
nga1139
nga1107
Chromosome 5
HY5
nga106
CIW8
PHYC
CIW9
CIW10

phenotypically the strongest of four *ted1* mutant alleles.

An Allele from Wild Type Bensheim (Be-0) Can Suppress *det1* Mutations

To determine whether wild type Be-0 carried an allele that would suppress the dark-grown phenotype of the *det1* mutation, we crossed wild type Be-0 with the homozygous *det1-1* mutant (isolated in the Col-0 genetic background). The *det1-1* mutation causes a splicing defect that results in a greatly reduced level of properly spliced transcript (Pepper *et al*., 1994). When the F_2 seedlings from the Be-0 x *det1-1* cross were examined as dark-grown seedlings, we observed a range of novel phenotypes that were intermediate between those of the *det1-1* and Be-0 parental controls. These 'partially etiolated' phenotypes were not observed in control F_2 seedlings from Col-0 x *det1-1* and Col-0 x Be-0 crosses. However, similar progeny were observed in a cross of Be-0 to a line that was homozygous for the *det1-4* allele (a hypomorphic missense mutation; Pepper *et al*., 1994).

Twelve seedlings representing a range of partially etiolated phenotypes were identified from each F_2 population, then propagated and allowed to self-fertilize. Using PCR-based diagnostic markers (Pepper and Chory, 1997) all 12 were determined to be homozygous for the *det1* mutant (*det1-1* or *det1-4*, respectively). In addition, all of the individuals were either homozygous or heterozygous for the Be-0 allele of CAPS marker SC5, located on chromosome 4 in the vicinity of the putative QTL for light-grown hypocotyl length. The possibility of the latter result occurring by chance was excluded by χ^2 analysis ($\chi^2 = 8.0$, $p < 0.01$). Dark grown F_3 seedlings descended from two of the

'partially etiolated' F_2 seedlings (homozygous for *det1-1* and *det1-4*, respectively) with the most extreme long-hypocotyl phenotypes are shown in Figure 2.4. These show clear suppression of the *det1* phenotype.

In a separate experiment, the wild-type Be-0 line was crossed to the *ted1-1 det1-1* double mutant line (which showed partial suppression of the *det1* phenotype). In the F_2 generation from this cross, a wide range of dark-grown phenotypes was observed. However, typical *det1* progeny (similar to the *det1-1* controls) were not present among the approximately 1,090 seedlings examined. Considered together, these results are consistent with a model in which the wild type Be-0 accession carries an allele at the *ted1* locus that is functionally similar to the strongest allele of *ted1* (*ted1-1SD*) obtained through EMS-induced mutagenesis of the Col-0 ecotype.

In the F_2 generation of a Col-0 x *ted1-1SD det1-1* cross, we had previously observed progeny with light-grown hypocotyls that were longer than the Col-0 control. One of these extreme long-hypocotyl segregants was isolated and propagated by self-fertilization for several generations. In the F_7 generation, this line was likely to be homozygous at all loci affecting hypocotyl length. In the experiment shown in Figure 2.5, we compared the light responses of this F_7 population (designated F_7-CT) with those of Be-0 and Col-0. As shown, F_7-CT has similar hypocotyl growth responses to Be-0. The most significant differences with respect to Col-0 are observed in white light (45 μmol m^{-2} s^{-1}) and red light (23 μmol m^{-2} s^{-1}).

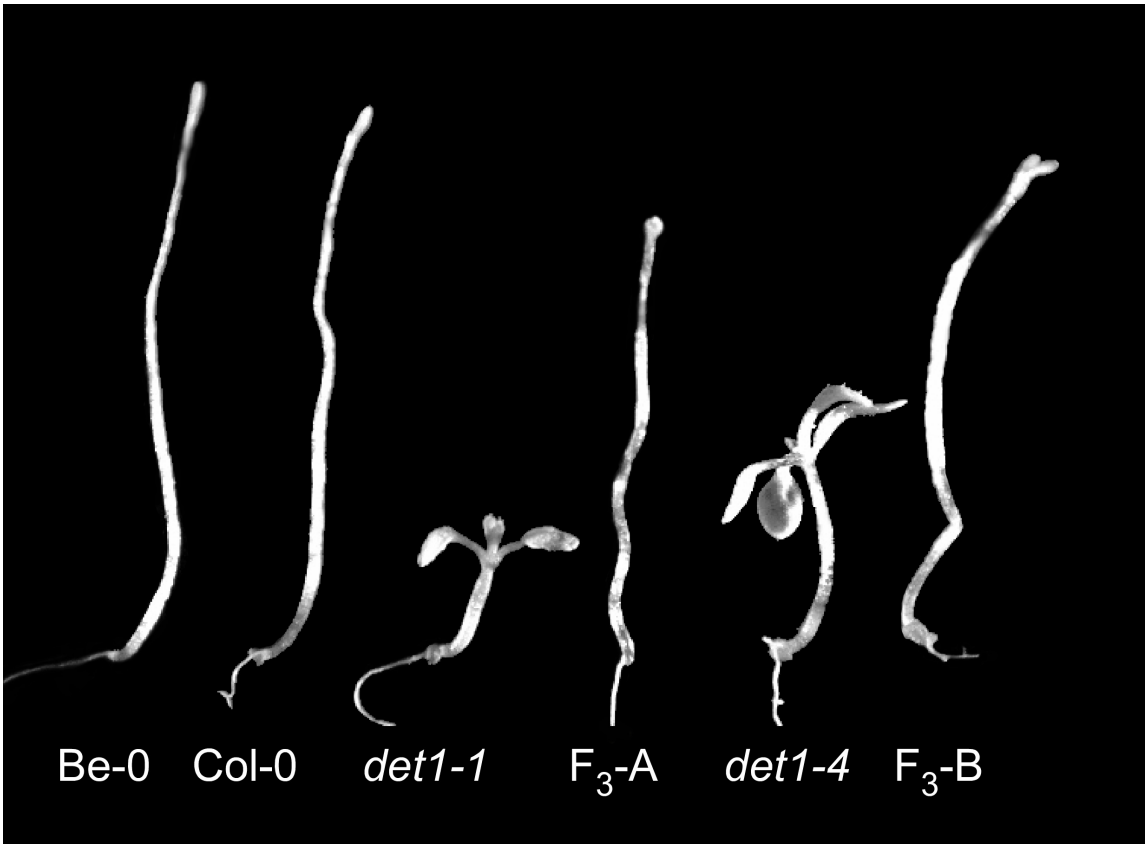


Figure 2.4. Morphologies of 8 day old dark-grown seedlings.

Be-0, Col-0, *det1-1* (isolated in the Col-0 genetic background) and *det1-4* (isolated in the Col-0 genetic background) are indicated. Seedling F_3-A was derived from a Be-0 x *det1-1* cross and is homozygous for the *det1-1* allele. Seedling F_3-B was derived from a Be-0 x *det1-4* cross and is homozygous for the *det1-4* allele. Both F_3-A and F_3-B are representative of the most extreme long-hypocotyl progeny from among those progeny that are homozygous for *det1-1* and *det1-4*, from their respective crosses.

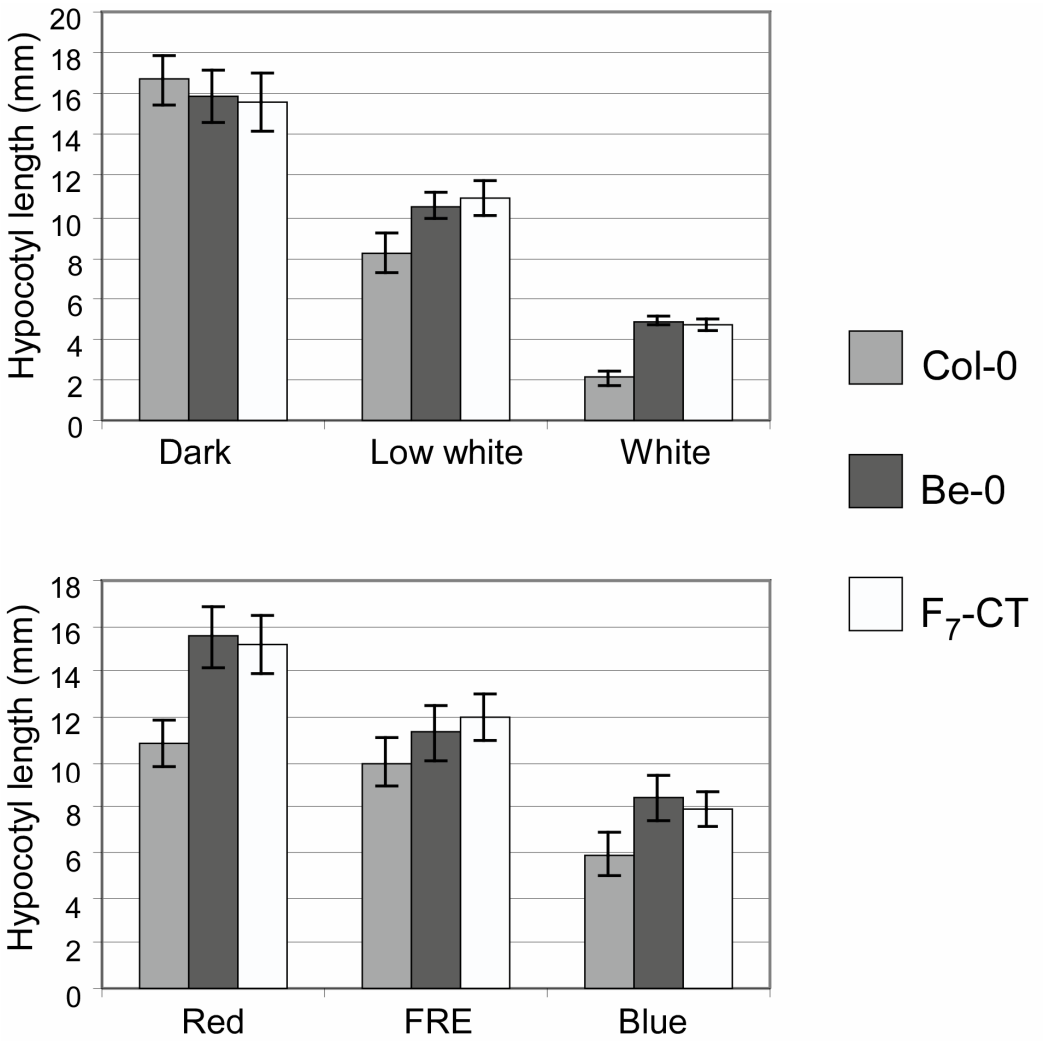


Figure 2.5. Comparison hypocotyl phenotypes of Be-0, Col-0 and an F_7 long-hypocotyl line descended from a cross of Col-0 to *ted1-1SD det1-1*.

Hypocotyl phenotypes of Be-0, Col-0 and an F_7 long-hypocotyl line descended from a cross of Col-0 to *ted1-1SD det1-1* (Col-0 genetic background), designated as F_7-CT. Light conditions used were as described in Fig. 2.1. Error bars indicate standard deviations.

DISCUSSION

Environments, and therefore selection regimes, vary from one location to another. This may explain why the genetic analysis of natural variation among Arabidopsis accessions has been particularly productive in studies of plant interactions with biotic and abiotic components of their environment (Alonso-Blanco and Koornneef, 2000). The light environment experienced by seedlings varies as a result of biotic factors, such as competition (Ballare *et al*., 1990; Weinig, 2000b), and abiotic factors such as soil composition (Kasperbauer and Hunt, 1992; Botto *et al*., 1998) and geographic location. Differences in the selective regimes imposed by the environment are likely to lead to variation in light responses through: 1) positive selection leading to adaptation to particular environments, and 2) the effects of genetic drift in environments where certain selective pressures may be relaxed.

In the present study, we uncovered substantial genetic variation in light-dependent development, as quantified by hypocotyl length, within a relatively small set of Arabidopsis accessions collected from diverse edaphic habitats and geographic locations. Columbia (Col-0) is the most extensively utilized accession in laboratory experiments, including induced mutation studies, genome sequence analysis and functional genomics experiments. It is therefore of interest to note that Col-0 appears to be among the most light-responsive of the accessions, particularly in blue and white light.

The genetic variation we observed among accessions appears to be quite idiosyncratic in its spectral dependence (see Figure 2.1D). It is therefore likely that the

remarkable variation in light responses observed in our collection is conditioned by genetic diversity at several loci. In an independent survey of Arabidopsis accessions, Maloof *et al*. (2000) discovered a natural mutation in the hinge region of the *PHYA* gene in the Le Mans (Lm-2) accession leading to a substantial loss of PHYA function. Similarly, the individual or individuals from which the Wassilewskija (Ws-0) accession was derived also appear to have survived without a functional *PHYD* gene, although it is not known how wide-spread this loss-of-function allele of *PHYD* is in nature. Natural variation also occurs in genes that act downstream of the primary photoreceptors, as has been shown by Yanofsky *et al*. (1997) with the identification of *VLF1* and *VLF2*. The lack of clear spectral dependence in the phenotypic variation observed in our collection suggests that the underlying genetic variation may be polygenic in nature, with different genes having effects on different signaling pathways. Alternatively, the variation may reside in downstream components of light signaling pathways that play roles in the perception of both red and blue light. Clearly, natural variation in photomorphogenic responses (including floral initiation, germination and vegetative morphology) is an expansive source of new variant alleles for studies of gene function at both the molecular and population-genetic levels.

In the present study, we discovered that the substantial differences in hypocotyl responses to white light between Col-0 and Be-0 mapped to single major gene located near the middle of chromosome 4. For this analysis, we used a set of framework PCR-based markers that we routinely use for the genetic mapping of newly identified loci. Because this framework marker set included several markers derived from, or closely

linked to known photomorphogenetic regulatory genes, we could identify 'candidates' for genes underlying natural variation in photomorphogenetic responses in the F_2 generation. The most significant signal from QTL analysis implicated a genetic interval that contains the *PHYD* and *PHYE* apoprotein genes, as well as the uncloned *TED1* gene. Since the genetic differences between Col-0 and Be-0 have strong phenotypic manifestations in red light as well as white light (Figures 2.1B and 2.5), *PHYD* and *PHYE* would seem reasonable candidates for the gene underlying these differences. However, apparent null or near null alleles of each of these genes have subtle effects, if any, on light-grown seedling morphology, unless in the presence of a loss-of-function mutation in *PHYB* (Aukerman *et al*., 1997; Devlin *et al*., 1998; Devlin *et al*., 1999).

It is possible that the *ted1-1SD* mutation, as well as the natural allele present in Be-0, might be novel and functionally distinctive alleles of *PHYD* or *PHYE* that encode a gene product that gives rise to phenotypic effects in both red and blue light (Figure 2.5). The precise relationship of the *TED1* locus to the *PHYD* and *PHYE* genes is unknown. The best available mapping data suggests that the *ted1* locus is located between the *PHYD* and *PHYE* genes (R.W. Corbett & A.E. Pepper, unpublished). The Be-0 accession does not carry the 14 bp *PHYD* deletion mutation present in Wassilewskija (data not shown). However, both *PHYD* and *PHYE* apoprotein genes remain plausible candidates for *TED1*.

We previously observed that the *ted1-1SD det1-1* mutant line gave rise to progeny with a moderately long hypocotyl in white light when outcrossed into the wild type (*DET1*) genetic background. Since this effect was only observed in one of four

alleles of *ted1*, it could be argued — justifiably — that the effect may not be due to the *ted1* suppressor locus, but rather to a second mutation in a closely linked gene. In the present study we uncovered several independent lines of evidence indicating that the natural Be-0 accession may contain a variant allele at the *TED1* locus, functionally similar to *ted1-1SD*, that gives rise to a moderately long hypocotyl phenotype in the light (relative to Col-0) and suppresses the *det1* phenotype in the dark. Thus, we are now likely to have two independently-derived alleles of *ted1* that show both the capacity to partially suppress the *det1* mutation, and a deficiency in hypocotyl responses to light. The deficiency in responses to red light as well as white light, seen in both the *ted1-1SD* derived line F_7-CT and in Be-0 (Figure 2.5), provides strong evidence for the involvement of this locus in some aspect of phytochrome signaling. This finding defines a role for *TED1* in light-signaling in the wild type (*DET1*) genetic background, and establishes a functional connection between phytochrome and *DET1* gene function.

CHAPTER III

NATURAL GENETIC VARIATION AT THE *TED1* LOCUS

OVERVIEW

Large-scale genomic methods were developed to assist in refining the position for *ted1*, a semi-dominant extragenic suppressor of the photomorphogenetic mutant *det1*. A natural long hypocotyl QTL and a locus suppressing the dark-grown phenotype of the *de-etiolated* mutant *det1* present in the Bensheim (Be-0) ecotype were both mapped to a single locus, *HAT4* (a transcription factor involved in shade-avoidance responses). Two functionally distinct alleles of *HAT4* in the Be-0 genetic background as well as multiple synonymous polymorphisms discovered via sequencing suggest that natural variation at the *HAT4* locus is responsible for much of the variation seen in hypocotyl length in naturally occurring ecotypes throughout the world. Additional experiments revealed a previously unknown genetic (and possibly molecular) interaction between *DET1* and *HAT4*. These findings suggest an important role for *HAT4* in seedling photomorphogenesis, in addition to its already known role in shade-avoidance responses. Models for the mechanism of action of suppression of *det1* by *HAT4* (*ted1*) are presented.

INTRODUCTION

In order to further our understanding of the role of *DET1* in photomorphogenesis, extragenic suppressor mutations were isolated that partially or fully restored the dark-grown phenotype of a *det1-1* homozygous mutant to a wild-type etiolated phenotype. The *det1-1* mutation is a G to A transition located just inside intron 1, five nucleotides from the 5' splice junction, leading to a defect in splicing of intron 1 (Pepper *et al*., 1994). The *ted1-1SD* mutation was originally isolated after a screen of *det1-1* homozygous mutant seedlings following an EMS (ethyl methanesulfonate) mutagenesis. *TED1* was roughly mapped to a ± 32 cM region between *DET1* and *AG* on chromosome IV (Pepper and Chory, 1997). The *ted1-1SD* mutation suppresses the dark-grown phenotype of *det1-1* in a semi-dominant manner; however it does not restore correct splicing of the *det1-1* transcript. In addition, *ted1-1SD* also suppresses or partially suppresses other alleles of *det1* including *det1-4* (a missense mutation) and *det1-6* (a null mutation) (Pepper and Chory, 1997).

In the previous chapter, an examination of natural genetic variation in seedling photomorphogenic responses uncovered a QTL underlying differences in light-grown hypocotyl length between the Columbia (Col-0) and Bensheim (Be-0) ecotypes of Arabidopsis. We mapped this QTL to a 4.8 cM interval between the CAPS marker SC5 and SSLP marker T6K21, within the same region of chromosome IV that contains the *ted1-1SD* mutation. Further, it was also shown that one or more loci from the wild-type Be-0 ecotypic background suppressed the *det1-1* dark-grown phenotype. At least one of these loci was very closely linked in repulsion phase to the *TED1* EMS allele in Col-0.

Based on these results, we proposed that the wild-type Be-0 ecotype carries a functionally distinct allele of *TED1* that suppresses *det1-1* and confers a long hypocotyl phenotype in the *DET1* background in the light (Pepper and Chory, 1997).

The overall goals of this project were to fine map, clone and characterize the putative novel allele of *TED1* in the Be-0 ecotype. To this end, new genomic methods were developed and employed in order to reduce the amount of time and effort required for processing large numbers of samples usually required for map-based cloning of QTLs (Borevitz and Chory, 2004). Methods developed here were used to identify the gene underlying the long hypocotyl QTL and to characterize the genetic variation at the putative *TED1* locus in the Be-0 ecotype.

MATERIALS AND METHODS

Plant Materials

Arabidopsis lines used in this study were single-seed descent lines from seeds stocks obtained from the Arabidopsis Biological Resource Center (ARBC) at Ohio State University or the laboratory stocks of Joanne Chory (Table 3.1). Single-seed descent lines were created by harvesting seeds from individual plants. Seeds used for phenotypic analysis were obtained from plants grown in a temperature-controlled growth room (24 ± 1 °C) under long day conditions (16 hours light / 8 hours dark) at a fluence rate of ± 100 μmol m^{-2} s^{-1}. Seeds were harvested from individual plants when the majority of the siliques were yellow / brown and prior to dehiscence. After collection, seeds were dried at low humidity for a minimum of 21 days prior to germination.

Table 3.1. Plant materials used for mapping *TED1*.

Strain	Ecotype / Accession	Source (ABRC no.)	Origin
Columbia (*DET1* wild type)	Col-0	J. Chory	Landsberg, Germany (Redei 1993)
det1-1	Col-0	J. Chory	Chory *et al*. (1989); Pepper *et al*. 1994)
Bensheim	Be-0	ABRC (CS6613)	Bensheim, Germany

Two different mapping populations were used to map the putative *TED1* allele in the Bensheim (Be-0) ecotype (Table 3.2). Both mapping populations were developed based on studies showing that the Be-0 likely carries an allele of *TED1* that can suppress the *det1-1* phenotype (Pepper *et al*., 2002).

The F_2(TAM826)A population: Be-0 × *det1-1* (Col-0)

Previously, Pepper *et al*. (2002) showed that the Be-0 ecotype probably carried a natural allele of *TED1* that suppressed the dark-grown phenotype of the *det1-1* mutant (Pepper *et al*., 2002). To further fine map this suppressor locus, a large mapping population was created by crossing Be-0 (male parent) to *det1-1* (female parent). The hybrid genotype of F_1 progeny was confirmed by testing for heterozygosity at the *DET1* locus using a Cleaved Amplified Polymorphic Sequence (CAPS) marker (Konieczny and Ausubel, 1993) specific for the *det1-1* mutation (Pepper and Chory, 1997). Any individuals that were derived from self-pollination of *det1-1* were excluded from further analysis. For this marker, a 117 bp fragment containing the *det1-1* mutation was

Table 3.2. Crosses used for mapping Be-0 allele of *TED1*.

TAMU Registry Number	Male Parent	Female Parent
F_2(TAM826)A	Be-0-1	*det1-1* (Col-0)
F_2(TAM793)C	Col-0	Be-0-1-01

amplified using primers 13-1G (5'-CTTCGAACGTCAGATTCGAACTCCTC-3') and 13-1revB (5'-CATTGAAGGTAA AGAGATAAGC-3'), then tested for cleavage by the restriction endonuclease *Bsm*FI. The PCR fragment amplified from an individual carrying *det1-1* allele will be cut by *Bsm*FI while an individual carrying the *DET1* allele will not. F_1 seedlings heterozygous for the *det1-1* mutation were allowed to self-pollinate to produce an F_2 mapping population which was then screened using the *det1-1* mutation specific CAPS marker. Since the trait being measured in this population was suppression of the *det1-1* phenotype, only those individuals that were homozygous for the *det1-1* mutation were included in the phenotypic and genotypic analysis.

The F_2(TAM793)C population: *Be-0 × Col-0*

In the previous chapter, a long hypocotyl QTL mapped to the same region of chromosome IV to which the original EMS suppressor mutant *ted1-1SD* was roughly mapped (Pepper and Chory, 1997; Pepper *et al*., 2002). To further refine the position of the gene(s) involved in the long hypocotyl QTL, an additional F_2 mapping population was created for mapping the Be-0 long hypocotyl QTL by crossing the Columbia (Col-0) ecotype to Be-0. F_1 hybrids were confirmed by screening individuals with the SSLP marker T6K21 developed in our lab. An F_2 population was derived by selfing of an F_1

individual heterozygous for both parental alleles at the T6K21 marker. In this population all F_2 individuals could be used for mapping (versus approximately 25% of the Be-0 × *det1-1* population discussed previously) because there was no need to screen for the *det1-1* mutation and select a sub-population for mapping.

Hypocotyl Measurements

Hypocotyl length is a well established experimental trait for the quantification of photomorphogenetic development (Pepper and Chory, 1997; Maloof *et al*., 2001; Botto and Smith, 2002; Pepper *et al*., 2002; Chen *et al*., 2004). Seeds for each mapping population, along with the parents of the F_1 (the parental controls), were sterilized as described in Chory, *et al*. (1989). Seeds were placed in 1.5 ml Eppendorf tubes and soaked in pure ethanol for one minute. Afterwards, the ethanol was removed by aspiration and the seeds were then soaked in a 2:1, sterile water to bleach solution containing the surfactant Tween 20 for ten minutes. Following bleach treatment, seeds were washed three to four times with sterile water and resuspended in a 0.1% phytagar solution. Seeds were cold treated by incubation at 4 °C for three to four days before plating on growth medium. Growth medium for the hypocotyl measurements was prepared by dispersing 35 mL of MS-2% medium (1 X Murashige and Skoog salts, 1 X Gamborg vitamins, 2% sucrose w/v, 0.8% phytagar w/v) (Murashige and Skoog, 1962; Gamborg *et al*., 1968) into 25 mm X 110 mm polystyrene Petri dishes. Plates were prepared one to two weeks before use in order to check for contamination of the medium before growing seeds.

Seeds were dispersed on the growth medium in a 5 mm grid pattern to ensure even spacing and exposed to four hours of white light (100 μmol m^{-2} s^{-1}) prior to treatments to initiate maximal and synchronous germination. Plates were rotated and shuffled under the light sources on a daily basis in order to reduce micro-environmental effects on phenotype within and between plates.

To map the long hypocotyl QTL, F_2(TAM793)C seedlings were grown under moderately low white light (40 μmol m^{-2} s^{-1}). This light level resulted in the greatest phenotypic discrimination between the wild-type Col-0 and Be-0 ecotypes based on hypocotyl length, as shown in Chapter II (Figures 2.1, 2.2). Seedlings were grown in either of two locations. The first set of measurements were taken from seedlings grown in an environmentally controlled growth chamber with light provided by a mixture of fluorescent lighting provided by four, 110 watt GE Cool-White fluorescent bulbs (F48T12-CW-1500) and incandescent lighting provided by two, 15 watt Sylvania Soft-White incandescent bulbs all of which filtered through two layers of gray, nylon mesh screen in order to lower the light intensity level. In the repeat experiment, seedlings where grown in converted office space with metal shelving and fluorescent light fixtures. In this experiment, seedlings were again grown under moderately low white light, however the light was provided by a combination of two, 40 watt Sylvania Gro-Lux fluorescent bulbs (F40/GRO/WS) and two, 34 watt GE Cool-White fluorescent bulbs (F40CW-RS-WM) filtered through a neutral density filter (Lee Filter #209) supported on a 2 mm thick piece of Lexan™. A filter was used for the repeat experiment in order to eliminate any ‘hot spots’ where light could travel straight through the openings in the

gray vinyl screen causing an individual to perceive a greater quantity of light than its siblings.

After eight days, hypocotyls of seedlings were measured under a stereo dissecting microscope using a 0.5 mm scale ruler (Ted Pella, Inc. product # 5448) in a laminar flow hood (Figure 3.1). Hypocotyls were gently straightened using a pair of No. 5 forceps and held against the ruler under the microscope. For those individuals whose hypocotyl length did not exactly fall on a 0.5 mm mark, a best estimate was recorded between the marks above and below. Measurements were recorded directly into an Excel spreadsheet using a numeric keypad while using an audible read-back for confirmation. After measuring, seedlings were transferred to numbered positions on fresh MS- 2% sucrose media plates to allow continued growth before DNA isolation.

To map the putative suppressor of *det1*-1, F_2(TAM826)A seedlings were grown under dark conditions in order to observe the suppression of the *det1-1* dark phenotype. Two replicate sets of measurements were taken in order to ascertain the reproducibility of the phenotypic data and to increase the effective mapping population size (the mapping populations excluded any individuals that were not homozygous for the *det1-1* mutation).

Dark-grown seedlings provide very little tissue for DNA extraction compared to seedlings grown under light. Because a large proportion of seedlings will not recover after seven days in the dark, callus culture was used to generate adequate quantities of tissue for DNA genotyping. After measuring, F_2(TAM826)A seedlings were transferred to numbered positions on a callus induction medium (CIM) (1 X Murashige and Skoog

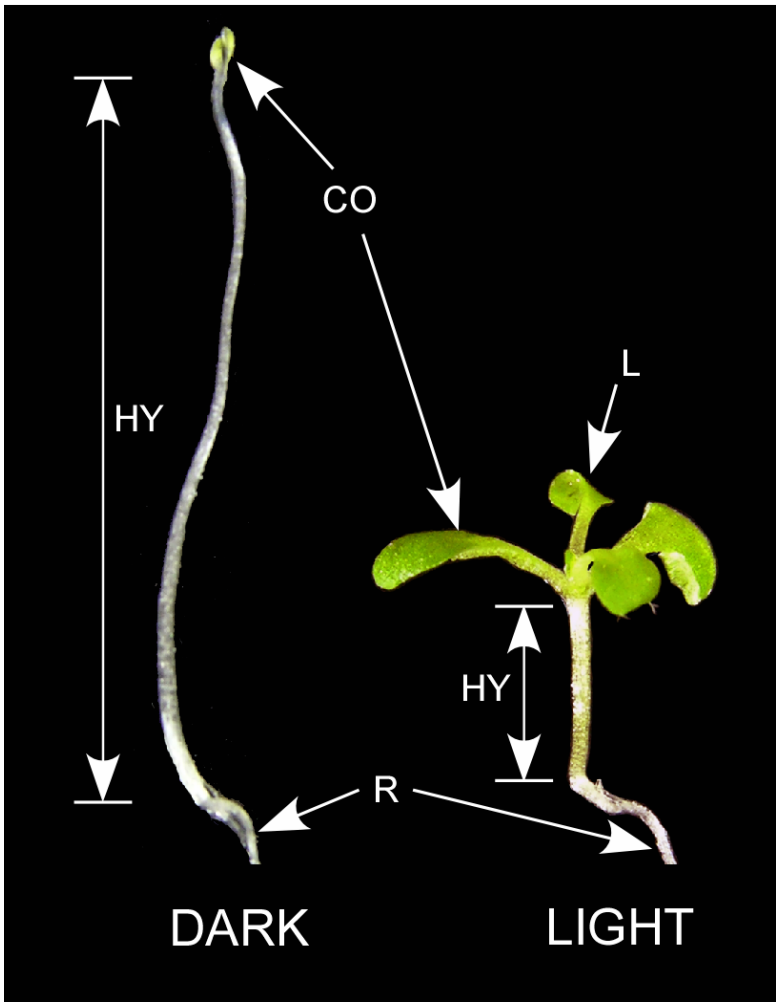


Figure 3.1. Phenotypic examples of dark and light grown Arabidopsis seedlings.

Phenotypes seen in the Col-0 "wild-type" ecotype of Arabidopsis under dark and light grown conditions. (CO) cotyledon, (L) true leaf, (R) root and (HY) hypocotyl are labeled. Hypocotyl length was used as a quantitative trait to measure photomorphogenetic responses.

salts, 1 X Gamborg vitamins, 3% sucrose w/v, 0.8% phytagar w/v, 1.0 mg / L 6-benzylaminopurine (BAP), 0.1 mg / L 1-naphthaleneacetic acid (NAA) (Patton and Meinke, 1988) to produce callus tissue for DNA isolation.

F_2(TAM793)C seedlings were placed on fresh MS-2% sucrose media and allowed to grow for an additional week under white light (100 μmol m^{-2} s^{-1}) before DNA was extracted. F_2(TAM826)A callus cultures were maintained for two to four weeks after initiation to allow for sufficient material to develop in order to produce enough DNA for mapping purposes.

Analysis of hypocotyl length to determine genetic and environmental effects on phenotype

Seeds from single-seed descent lines Col-0-7, Be-0-1-01, Be-0-1-2-8 and *det1-1* were sterilized and plated on MS-2% sucrose plates divided into 4 quadrants allowing all four genotypes to be placed on a single plate (~35 seeds per quadrant). Two replicate plates were used for each light environment analyzed (Table 3.3). After exposure to 4 hours of bright white light (~100 μmol m^{-2} s^{-1}) plates were placed in different light environments as well as in the dark. After 8 days, hypocotyls were measured and representatives of the genetic backgrounds from each light environment were photographed. Spectral outputs of each light environment were analyzed using a portable spectrophotometer (OceanOptics USB-2000, OceanOptics, Dunedin, FL) provided by the lab of Dr. Vincent Cassone, Department of Biology, Texas A&M University (Appendix A). Fluence rates of medium white, red enriched and blue light were

Table 3.3. Environments used for testing genetic and environmental effects on phenotype.

Light Environment	Location	Light Source	Light Quantity	Filters
Dark	Wrapped in foil	N.A.	N.A.	N.A.
Medium, white light	Growth room	(2) GE Cool White (FW40CW-RS-WM) 34 W (2) Sylvania Gro Lux (F40/GRO/AQ/WS/RP) 40 W	40 μmol m^{-2} s^{-1}	2 mm Lexan
Red-enriched light	Percival growth chamber	Quantum Devices LED array model SL515-670	20 μmol m^{-2} s^{-1}	N.A.
Far-red enriched light	Percival growth chamber	Quantum Devices LED array model SL515-735	20 μmol m^{-2} s^{-1}	N.A.
Blue light	Percival growth chamber	(4) Coralife® Actinic Blue 7100K (F20 T12 360^{0} BP) 20 W	15 μmol m^{-2} s^{-1}	Kopp 5-57 glass Lee #209 N.D.

measured with a quantum photometer (model LI-189, LI-COR, Lincoln, NE). Fluence rates of far-red enriched light were measured using a radiometer (model IL400, International Light, Newburyport, MA) with a far-red probed (model SEL033, International Light).

Analysis of hypocotyl growth

Representative individuals from each genetic background grown under the medium white light environment (40 μmol m^{-2} s^{-1}) were also used to make agarose impressions of their hypocotyls to observe epidermal cell size and shape (Mathur and Koncz, 1997). Hypocotyls were dissected from seedlings and placed on a film of 3% low melting-point agarose on a glass slide. After allowing the agarose to solidify at 4 °C for five minutes, hypocotyls were removed from the agarose and images of the impression were photographed using a Zeiss M^2BIO fluorescence combination zoom stereo/compound microscope equipped with a Zeiss AxioCam color digital camera. Images of the hypocotyl impressions were captured using the Zeiss AxioVision software version 3.0.6. A hemacytometer was photographed using the same magnification in order to convert pixels to actual μm. Images of hypocotyl cells were analyzed using the measurement tool in Adobe Photoshop 7.0. Measurements for length were taken from the upper left corner to the lower right corner of the cell since most cells in the hypocotyl were not perfectly square. Width measurements were taken at the widest point along the middle of the cell length. The ratio of cell height to width for each genetic background was used for comparisons due to differences in cell sizes along the hypocotyl.

DNA Extraction Methods

In the early part of this project, DNA was extracted from individuals in mapping populations using a modification of the method described by Pepper and Chory (1997). Either individual seedlings or calli were placed in a 1.5 ml Eppendorf tube and ground with a polypropylene pestle until the tissue was broken down before 0.5 ml of extraction buffer (200 mM Tris pH 7.5, 250 mM NaCl, 25 mM EDTA pH 8.0, 0.5% SDS) was added. After addition of the extraction buffer, samples were further ground until the tissue was broken down completely. Samples were centrifuged for four minutes at 16,000 × g (14,000 rpm) in a tabletop Eppendorf 5415C centrifuge to pellet the cellular debris and 450 µl of the supernatant was transferred to a new 1.5 ml Eppendorf tube containing 450 µl of isopropanol. Tubes were inverted to mix and the DNA was precipitated for ten minutes at room temperature. Following precipitation, samples were centrifuged for four minutes at 16,000 × g to pellet the DNA and the supernatant was decanted off of the resulting pellet. DNA pellets were resuspended in 50 mM Tris pH 7.5, 10 mM EDTA pH 8.0 using another polypropylene pestle and centrifuged an additional four minutes to remove any solids that did not dissolve. Afterwards, the DNA was precipitated a second time by transferring 450 µl of the supernatant into a new Eppendorf tube containing 50 µl 3 M sodium acetate, pH 5.2 and adding 500 µl isopropanol. The resulting pellets were washed with 70% ethanol to remove salts (Na, EDTA) that could possibly inhibit downstream processes. DNA pellets were dried in a Savant DNA110 Speed Vac, resuspended in 0.1 X TE (1 mM Tris pH 7.5, 0.1 mM EDTA pH 8.0), and stored at -20 °C until use.

High through-put (HTP) DNA extraction

It became apparent that DNA extraction from large numbers of individuals in our mapping populations was a major rate-limiting step of this project. In order to speed up the mapping process, new methods of DNA extraction were developed. The first method involved using a Black and Decker™ 9.6V cordless drill to grind the samples using the previous method. While this procedure did speed up the process, the time involved was still considerable because of the sample and reagent transfer steps using a single-channel micropipettor.

A 96-well high through-put (HTP) DNA extraction method for Arabidopsis was loosely modeled after the "paint shaker" method of Michaels and Amasino (2001). This and similar methods allow simultaneous DNA extractions of up to 96 samples at the same time. In our method, individual seedlings or callus cultures were placed in separate tubes of a Costar macro-tube library plate (Costar part #4413). A 1/8" X 1/2" stainless steel bar (Small Parts Inc., Miami Lakes, Florida, part #DWX-02-08-C) and 500 µl of DNA extraction buffer (200 mM Tris pH 7.5, 250 mM NaCl, 25 mM EDTA pH 8.0, 0.5% SDS) was added to each macro tube and the entire plate was sealed with strip caps (Costar part #4418). Tissue was ground for 2 minutes in a Spex Centriprep GenoGrinder 2000 set at 1500 strokes per minute. A brief spin at 3250 rpm in a Beckman GS-6KR centrifuge equipped with a PTS-2000 rotor was done in order to remove droplets from the strip-caps before proceeding. Cellular debris were removed by transferring 400 µl of supernatant from each DNA sample into a 96-well, 0.45 µm, melt-blown polypropylene filter plate (Phenix research products, part # F-20005) seated above a 1 ml, 96-well

receiving plate (Phenix research products, part # M-0355) containing 400 µl isopropyl alcohol per well. The assembly was then centrifuged in a Beckman GS-6KR centrifuge at 1,920 × g (3,250 rpm). Samples were mixed by pipetting and left at room temperature for ten minutes to precipitate the DNA and then centrifuged at 1,920 × g (3,250 rpm) for thirty minutes to pellet the DNA. The supernatant was removed as quickly as possible after centrifugation in order to prevent loosening of the pellet by inverting the plate over a sink and then placing the plate on a stack of clean paper towels to allow for residual liquid to drain off. After ten minutes, plates containing the DNA pellets were dried to completion using a Savant SC210A Speed Vac Plus and then resuspended in 50 to 100 µl 0.1 X TE (50 mM Tris-HCl, pH 7.5; 0.1 mM EDTA, pH 8.0) depending on the amount of starting tissue. The quality and quantity of DNA isolated from the HTP DNA extraction was examined by running 5 µl of each sample on a 1% agarose gel using standard procedures. Samples were treated with 0.1 µg/ml RNase for one hour and stored at -20 °C until use.

Molecular Marker Development and Genetic Mapping

Simple sequence length polymorphism (SSLP) (Bell and Ecker, 1994) and cleaved amplified polymorphic sequence (CAPS) (Konieczny and Ausubel, 1993) marker methods used for quantitative trait locus (QTL) mapping were developed from polymorphism sequence and information obtained from The Arabidopsis Information Resource (TAIR) website (http://www.arabidopsis.org) or were designed from sequence data generated in our laboratory. Markers with inter-ecotypic polymorphisms between

the Col-0 and Landsberg-*erecta* (La-*er*) ecotypes obtained from the TAIR web site (http://www.arabidopsis.org) were tested for polymorphism in the Bensheim ecotype. Additional SSLP markers were created by scanning the Columbia (Col-0) ecotype sequence data for SSLPs (also known as microsatellites) and other repetitive DNA structures. Primer pairs were then designed that would amplify regions containing these repetitive (and often polymorphic) sequences. In cases where markers were needed in areas without repetitive elements, sequence data was directly obtained from Be-0 fragments amplified by PCR for use in the design of CAPS markers. A full list of markers and their related information can be found in Appendix A. Individuals in the mapping population, along with genomic controls from the parents, were amplified with a set of polymorphic markers using standard PCR conditions (Bell and Ecker, 1994).

PCR fragments amplified from individuals were electrophoresed on 4% agarose gels (2% Metaphor™ agarose, 2% agarose) at 15 V cm^{-1} using a modified 0.5 X TBE (0.088 M Tris-borate, 0.001 M EDTA) as running buffer. Tabletop fans were used to circulate air around running chambers during electrophoresis to prevent overheating. Gels were stained with ethidium bromide after electrophoresis and visualized using an Alpha-Innotech gel documentation system. Molecular marker data and phenotype data were subjected to QTL analysis using the composite interval mapping (CIM) method (Jansen, 1993; Zeng, 1993, 1994) for the F_2(TAM793)C mapping population and the single marker analysis (SMA) method (Lander and Botstein, 1989) for the F_2(TAM826)A mapping population of QTL Cartographer (Wang et al., 2001-2004)

Sequence Analysis of Candidate Genes

Candidate genes for the long hypocotyl QTL/*TED1* were PCR amplified from genomic DNA isolated from individual seedlings of Col-0-7 and single-seed descent Be-0 lines Be-0-1-01 and Be-0-1-2-8, then sequenced directly. PCR fragments (0.9 to 3.2 Kbp) were amplified from genomic DNAs to be used as templates for direct sequencing. Initial amplifications from genomic DNA were performed in 100 µl reactions using an Applied Biosystems GeneAmp 9700 PCR machine and standard PCR conditions. After amplification, 4 µl of the PCR reaction was electrophoresed on a 1% agarose gel to check the quantity and quality of the PCR product. The remaining 96 µl was purified using a Qiagen PCR purification kit to remove unincorporated nucleotides and primers. Purified PCR products were used for cycle sequencing reactions with BigDye™ (Applied Biosystems) in a 10 µl PCR reaction using primers spaced at intervals that would produce overlapping sequencing products (500 to 800 bp) from one or both strands (Table 3.4). Sequencing reactions were purified by passing through Sephadex G-50 spin columns to remove unincorporated BigDye™ and dried to completion with a Savant DNA110 SpeedVac. Purified sequencing reactions were resuspended in 11 µl of de-ionized formamide and analyzed using an Applied Biosystems 3100 capillary DNA sequencer by personnel at the Laboratory for Plant Genome Technologies in the Norman Borlaug Center at Texas A&M University or the Gene Technologies Laboratory in the Department of Biology at Texas A&M University. Sequence reads were aligned and analyzed using Sequencher (GeneCodes, Ann Arbor, MI) to identify polymorphisms present in candidate genes and their 5' regulatory sequences that could possibly lead to

Table 3.4. Primers used for sequencing *HAT4*.

Primer	Sequence (5' > 3')	Position[a]
HAT4CL-F	CGAAAAGTTTGTGTGTCTGATTCC	-1954
HAT45U3-F	CGACCTTTATGACCAAAATGGGTC	-1634
HAT45U3-R	GACCCATTTTGGTCATAAAGGTCG	-1611
HAT45U2-F	CCATGCGTTAGAAGAAGAAAATCTG	-1080
HAT45U2-R	CAGATTTTCTTCTTCTAACGCATGG	-1056
HAT45U1-F	GACTCAACGATCTAACCAATCACC	-599
HAT45U1-R	GGTGATTGGTTAGATCGTTGAGTC	-576
HAT45U-R	CCCCTCTATATAACTCTCTCAAAC	-272
HAT4BAM-F	AGCTAGTCGGATCCTCATGATTG	-172
HAT4BAM-R	CAATCATGAGGATCCGACTAGC	-150
HAT4E1-R	GTCTTTCTCGAACATCATCTTCTG	18
HAT4EX1-F	AAGATCTTCATGGAACGAGAGTTTTAC	126
HAT4EX2-F	CGTCTACAGCGGAATACGGC	333
HAT4EX2-R	GCCGTATTCCGCTGTAGACG	352
HAT4EX2B-F	GTGACGATGAAGATGGTGATAACTCC	459
HAT4I2-F	TCACCCGAGAAAGTTTGGTTTTAC	575
HAT4I2-R	GTAAAACCAAACTTTCTCGGGTG	598
HAT4E3-R	GCTCGTAACCCTAATTGTTTAGCC	839
HAT4E3-F	AATTAGGGTTACGAGCAAGACAAG	854
HAT4RT-F	CCGTCGGCTACAAAAAGAAGTAACGGAATTG	1053
HAT4E4-F	CACGGCTTGACTTTTGACGCTC	1258
HAT4RT-R	GGTTGCAAGTAAAAAGACTTAGGACCTAGGAC	1314
HAT4SPE-R	GTTGTTACTAGTTAGCATGACAC	1609
HAT4CL-R	AGTAAATCCGACACCAAGCTGC	2164

[a] Measured in bp from 5' end of primer relative to the start of translation

altered gene function, and for use in the development of gene-specific markers.

Quantitative RT-PCR

In order to analyze the expression patterns of candidate genes for the long hypocotyl QTL/*TED1*, quantitative RT-PCR was performed on RNA samples isolated from Col-0, *det1-1*, and two single-seed descent Be-0 lines (Be-0-1-01 and Be-0-1-2-8). Seedlings where grown as previously described under moderate white light (40 μmol m^{-2} s^{-1}) and in the dark. Seedlings were also grown in narrow spectrum red enriched light provided by a red LED array (20 μmol m^{-2} s^{-1}) or far-red enriched light provided by a far-red LED array (20 μmol m^{-2} s^{-1}). After seven days, RNA was isolated from seedlings using a Qiagen Plant RNeasy RNA extraction kit. RNA concentration was determined using a Beckman DU60 Spectrophotometer and quality of the RNA was observed by agarose gel electrophoresis. Following quantification and DNAseI treatment (DNA-*free*™ kit, Ambion, Austin, TX) to remove genomic DNA, 700 ng of RNA was reverse transcribed using an Ambion RETRO-SCRIPT kit with random decamers.

Quantitative PCR was performed on the reversed transcribed cDNA using an Invitrogen SYBR quantitative PCR kit in conjunction with an ABI 7900HT real-time (RT) PCR machine. Expression levels of *EF1α, DET1* and candidate gene *HAT4* were quantified using cDNA from each genotype under all four light conditions (16 total combinations). RT-PCR products were amplified with gene specific primers for *DET1* (DET1RT-F, 5'- GCAGATGGTCGAAGCAAGAAGATA-3'; DET1RT-R, 5'- GATATT GACGACAGAAGGTGGCATG-3'; *HAT4* (HAT4RT-F, 5'-CCGTCGGCTACAAAAA

GAAGTAACGGAATTG-3'; HAT4RT-R 5'-GGTTGCAAGTAAAAAGACTTAGGAC CTAGGAC-3'). In addition to *DET1* and *HAT4,* the *EF1α* gene was amplified with gene specific primers (EF930-F, 5'-TCGAATCCTCAAAACTCTATCCGCA-3'; EF930-R, 5'-GGAGAAGAA ACGAAGCTATAACACG-3') as a constitutive control.

For quantitative RT-PCR, reactions containing SYBR green master mix (Invitrogen), cDNA, and gene-specific primers were mixed then heated at 94 °C for 10 minutes followed by 60 cycles of two-step PCR with a thermal profile of 95 °C for 10 seconds, 60 °C for 1 minute. To verify the amplification of single PCR products, a dissociation curve analysis was performed after cycling by denaturing PCR products at 95 °C for 30 seconds followed by annealing of PCR products at 60 °C for 30 seconds and a 2% ramping to 95 °C. Additionally, PCR products were electrophoresed on a 1% agarose gel to check for non-specific amplification products that would have resulted in false signal.

RESULTS

Fine Mapping of Long Hypocotyl QTL

Hypocotyl length distributions

Two independent platings of seeds for each experimental mapping population were used for hypocotyl measurements (Table 3.5). Hypocotyl lengths for both of the F_2(TAM793)C Col-0 × Be-0 mapping experiments showed a normal distribution encompassing the parental controls used to create the population (Figure 3.2) as seen previously (Pepper and Chory, 1997). All individuals measured could be genotyped for the purposes of mapping the long hypocotyl QTL allele.

Table 3.5. Summary of long hypocotyl QTL mapping populations.

Mapping population	$\mathbf{n_{(measured)}}$	$\mathbf{n_{(genotyped)}}$
F_2(TAM793)C	404	404
F_2(TAM793)C REPEAT	305	305

Fine mapping of long hypocotyl QTL

A first-pass mapping population of 404 individuals from F_2(TAM793)C was used to refine the position of the long hypocotyl QTL roughly mapped to a 32 cM (804 Kbp) between markers SC5 and T6K21 (Pepper *et al*., 2002) containing 190 protein coding sequences (www.arabidopsis.org). In this region, genes considered as strong candidates involved in the long hypocotyl QTL were the phytochrome *PHYD*, the homeobox domain-leucine zipper transcription factor *HAT4* (a gene involved in the

Figure 3.2. Hypocotyl length distributions for F_2(TAM793)C mapping population.

(A) Summary photograph depicting hypocotyl phenotypes seen in measured population (bar = 1 mm).

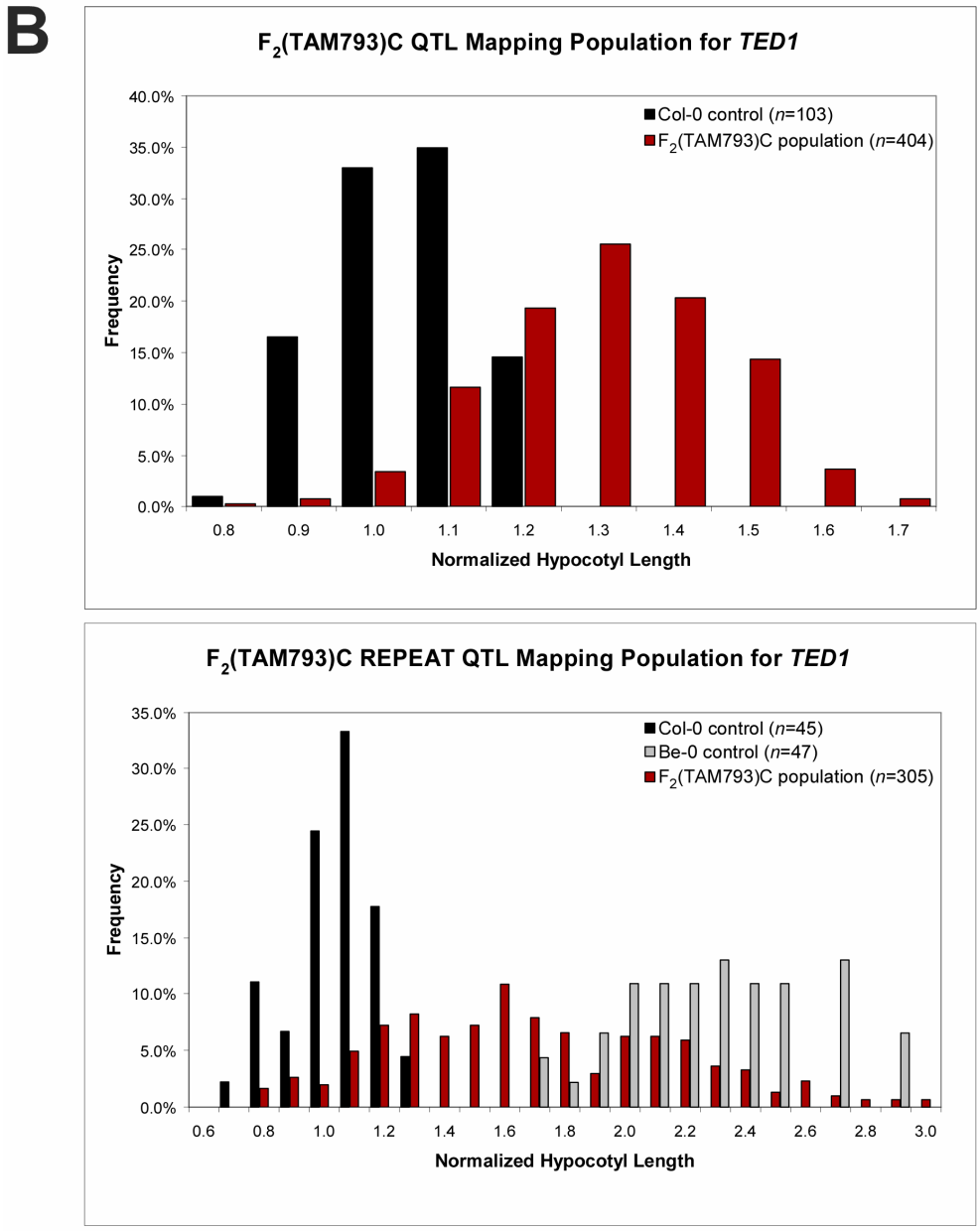


Figure 3.2. Continued.

(B) Hypocotyl length distribution for F_2(TAM793)C populations. Hypocotyl lengths were normalized on each Petri plate to the average Col-0 to eliminate microenvironmental effects on phenotypic data within and between plates. The initial population does not contain data for the Be-0 parental control.

shade avoidance responses and directly effecting hypocotyl length) (Carabelli *et al*., 1993; Carabelli *et al*., 1996; Steindler *et al*., 1999; Morelli and Ruberti, 2002) and *HAT1* (an ortholog of *HAT4*) (Schena and Davis, 1994).

Since the distribution of hypocotyl length in the mapping population had a roughly normal distribution (Figure 3.2), all individuals were able to be used for mapping purposes. Quantitative trait locus (QTL) mapping using the composite interval mapping (CIM) method (Jansen, 1993; Zeng, 1993, 1994) of QTL Cartographer version 2.0 (Wang *et al*., 2001-2004) was used to narrow the region for the long hypocotyl QTL in Be-0. The interval containing the *TED1* allele in the Be-0 ecotype was initially narrowed to a 371 Kbp region between the SSLP markers FCA6.7 and FCA8.3 (Figure 3.3) containing 80 predicted gene products (www.arabidopsis.org). The mapping data eliminated *PHYD* as a candidate for the gene underlying the long hypocotyl QTL.

Six individuals from the initial F_2(TAM793)C population were still recombinant (hybrid) at the boundaries of this 371 Kbp interval (between FCA6.7 and FCA8.3) and thus carried recombination breakpoints that could be informative for fine-mapping the QTL. In a repeat experiment, an additional 305 individuals were plated and measured for QTL analysis (Figure 3.2), another six individuals that were still recombinant at SSLP markers FCA6.7 and FCA8.3 were identified. Development of an additional SSLP marker, FCA7D2, narrowed the interval further from 371 Kbp to 191 Kbp between SSLP markers FCA6.7 and FCA7D2, which contained 38 predicted gene products (Table 3.6) and eliminated *HAT1* as a candidate gene. Within this interval, the highest LOD score (6.60) was found at a CAPS marker in the *HAT4* gene (Figure 3.3).

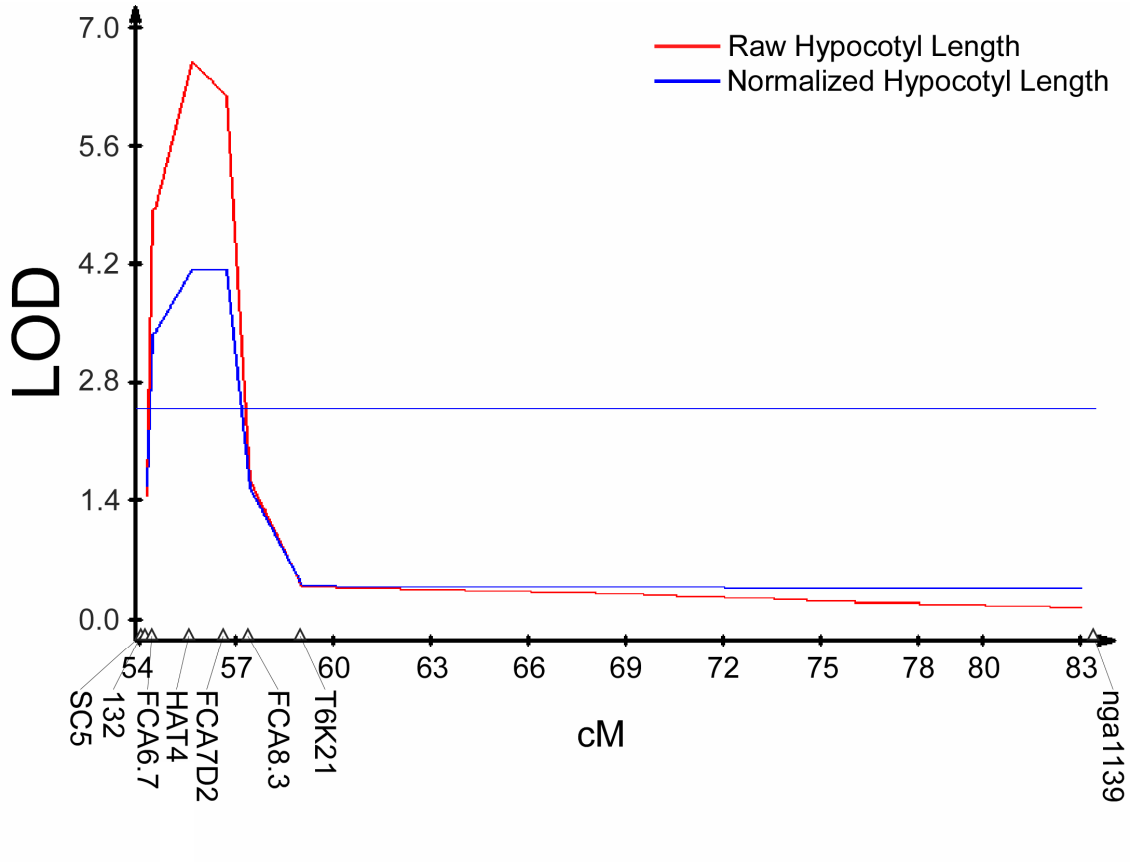


Figure 3.3. QTL Analysis for F_2(TAM793)C.

QTL Cartographer output for mapping of long hypocotyl QTL using composite interval mapping (CIM) in F_2(TAM793)C mapping population. Significance LOD = 2.5.

Table 3.6. Genes in interval between SSLP markers FCA6.7 and FCA7D2.

	Gene Model	Description
	AT4G16740.1	terpene synthase/cyclase family protein, similar to myrcene/ocimene synthase
	AT4G16745.1	exostosin family protein
	AT4G16750.1	DRE-binding transcription factor, putative, similar to DRE binding factor 2 (*Zea mays*)
	AT4G16760.1	acyl-CoA oxidase (ACX1), identical to acyl-CoA oxidase (*Arabidopsis thaliana*)
	AT4G16765.2	oxidoreductase, 2OG-Fe(II) oxygenase family protein, similar to *isp7* (*Schizosaccharomyces pombe*), flavanone-3-hydroxylase (*Medicago sativa*), flavanone 3-hydroxylase (*Perilla frutescens*)
	AT4G16770.1	oxidoreductase, 2OG-Fe(II) oxygenase family protein, low similarity to flavonol synthase (*Petunia hybrida*, *Citrus unshiu*)
*	AT4G16780.1	homeobox-leucine zipper protein 4 (HAT4) / HD-ZIP protein 4 (*Arabidopsis thaliana*) (HD-ZIP homeotic protein *Athb-2*)
	AT4G16790.1	hydroxyproline-rich glycoprotein family protein, contains proline-rich extensin domains
	AT4G16800.1	enoyl-CoA hydratase, putative, similar to AU-binding protein/Enoyl-CoA hydratase (*Homo sapiens*, *Mus musculus*)
	AT4G16810.1	expressed protein, similar to vernalization 2 protein (*Arabidopsis thaliana*), embryonic flower 2 (*Arabidopsis thaliana*), fertilization-independent seed 2 protein (*Arabidopsis thaliana*)
	AT4G16820.1	lipase class 3 family protein, similar to DEFECTIVE IN ANTHER DEHISCENCE1 (*Arabidopsis thaliana*)
	AT4G16830.1	nuclear RNA-binding protein (RGGA), identical to nuclear RNA binding protein (*Arabidopsis thaliana*)
	AT4G16835.1	pentatricopeptide (PPR) repeat-containing protein
	AT4G16840.1	Expressed protein
	AT4G16845.1	vernalization 2 protein (*VRN2*), identical to vernalization 2 protein (*Arabidopsis thaliana*)
	AT4G16850.1	expressed protein
	AT4G16860.1	disease resistance protein (TIR-NBS-LRR class), putative
	AT4G16880.1	disease resistance protein-related, contains weak similarity to disease resistance protein *RPP4* (*Arabidopsis thaliana*)
	AT4G16890.1	disease resistance protein (TIR-NBS-LRR class), putative
	AT4G16900.1	disease resistance protein (TIR-NBS-LRR class), putative
	AT4G16920.1	disease resistance protein (TIR-NBS-LRR class), putative
	AT4G16930.1	disease resistance protein (TIR-NBS-LRR class), putative
	AT4G16940.1	disease resistance protein (TIR-NBS-LRR class), putative
	AT4G16950.1	disease resistance protein (TIR-NBS-LRR class), putative; closest homolog in Col-0 to RPP5 of cultivar Landsberg *erecta*.

Table 3.6. Continued.

Gene Model	Description
AT4G16960.1	disease resistance protein (TIR-NBS-LRR class), putative
AT4G16970.1	protein kinase family protein, contains protein kinase domain
AT4G16980.1	arabinogalactan-protein family, similar to arabinogalactan protein (*Arabidopsis thaliana*); contains proline-rich extensin domains
AT4G16990.1	disease resistance protein (TIR-NBS class), putative
AT4G16990.2	disease resistance protein (TIR-NBS class), putative
AT4G16990.3	disease resistance protein (TIR-NBS class), putative
AT4G17000.1	hypothetical protein
AT4G17010.1	expressed protein
AT4G17020.1	transcription factor-related, contains weak similarity to TFIIH basal transcription factor complex p52 subunit (*Homo sapiens*)
AT4G17030.1	expansin-related, identical to expansin-related protein 1 precursor (*At-EXPR1*)(*Arabidopsis thaliana*)
AT4G17040.1	ATP-dependent Clp protease proteolytic subunit, putative (*Synechococcus* sp.PCC 7942)
AT4G17050.1	expressed protein
AT4G17060.1	expressed protein
AT4G17070.1	expressed protein

* candidate gene for *TED1*

Mapping the Suppressor of *det1-1* in Be-0

Hypocotyl length distributions

Unlike the F_2(TAM793)C mapping population, F_2(TAM826)A was used to map the Be-0 alleles of *TED1* that suppress the *det1-1* phenotype. Only those individuals homozygous for the *det1-1* mutation were used for mapping (approximately ¼ of the total number of individuals measured) (Table 3.7, Figure 3.4).

Table 3.7. Summary of *det1-1* suppressor in Be-0 mapping populations.

Mapping population	$\mathbf{n_{(measured)}}$	$\mathbf{n_{(det1\text{-}1/det1\text{-}1)}}$	$\mathbf{n_{(genotyped)}}$
F_2(TAM826)A	188	118	44
F_2(TAM826)A REPEAT	136	55	55

Fine mapping of det1-1 suppressor in Be-0

Due to the fact that individuals in the F_2(TAM826)A mapping population were screened for homozygosity of the *det1-1* mutation before mapping of *TED1*, standard methods of interval mapping (IM) and composite interval mapping (CIM) QTL analysis could not be used (because of the assumptions for QTL algorithms that populations have a normal distribution of phenotypes, and there be no selective bias for one phenotype over another). Out of 188 individuals measured for the initial experiment, 118 were homozygous for the *det1-1* mutation. An additional 136 individuals from a second

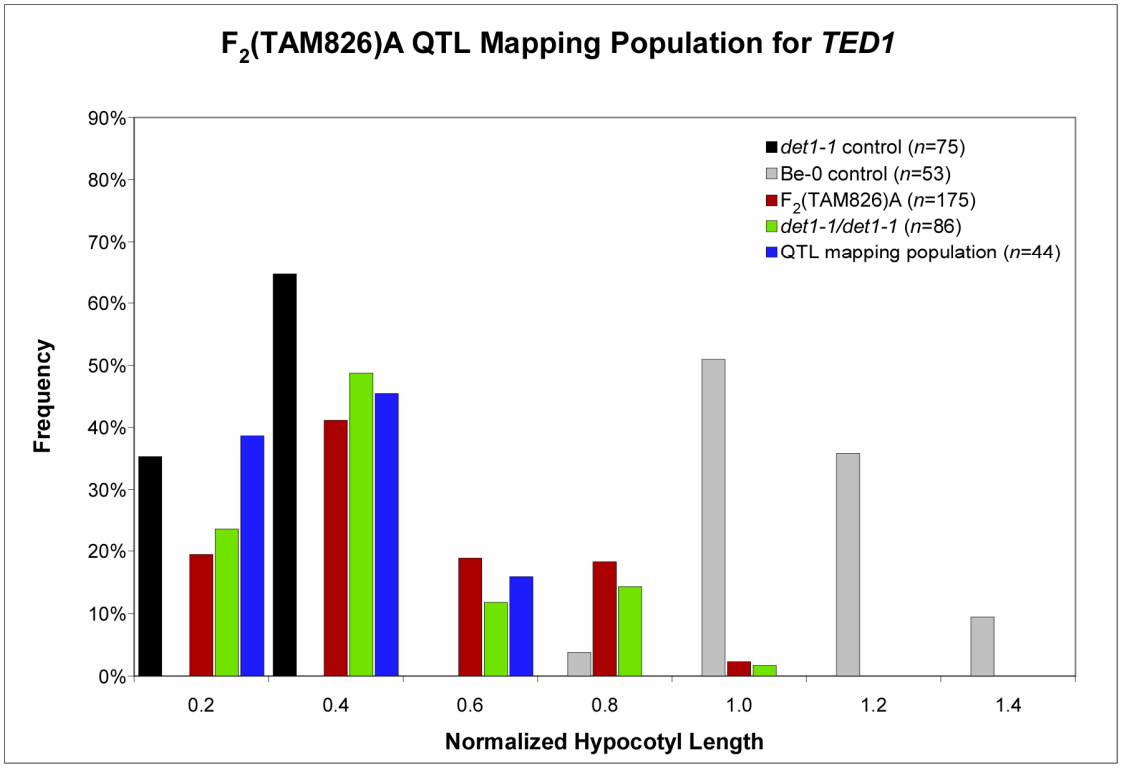


Figure 3.4. Hypocotyl length distributions for F_2(TAM826)A mapping population.

Hypocotyl lengths were normalized on each Petri plate to the average Be-0 to eliminate microenvironmental effects within and between plates on phenotypic quantitative data. Only individuals that were homozygous for the *det1-1* mutation were used for mapping of the Be-0 allele of *TED1*.

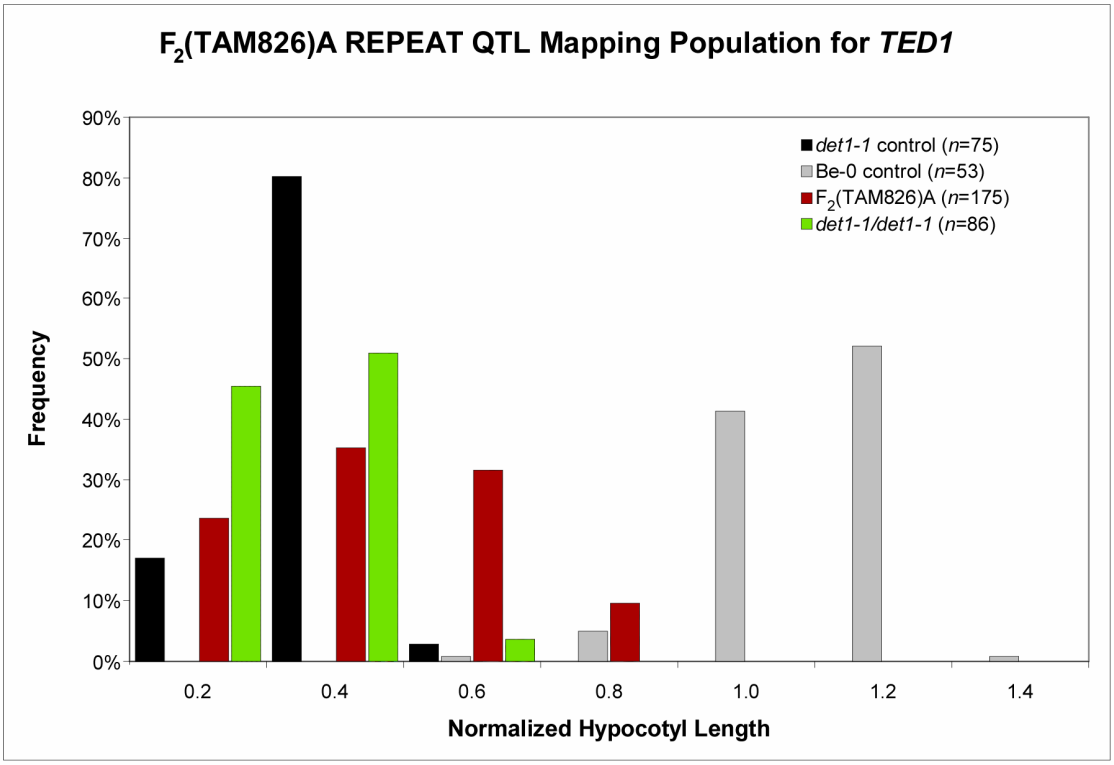


Figure 3.4. Continued.

Hypocotyl lengths were normalized on each Petri plate to the average Be-0 to eliminate microenvironmental effects between plates on phenotypic quantitative data. Only individuals that were homozygous for the *det1-1* mutation were used for mapping of the Be-0 allele of *TED1*.

experiment were measured, and 55 were homozygous for the *det1-1* mutation (Table 3.7).

Since interval or composite interval QTL mapping could not be used because of the selection of a subgroup from the overall population, single marker analysis (SMA) was used instead to determine the probability that a marker is linked to a QTL containing an allele of *TED1* in the Be-0 background (Table 3.8). Linkage to the CAPS marker HAT4 was significant ($P<0.0001$) for linkage to a QTL in the F_2(TAM826)A mapping population containing a putative allele of *TED1* that can suppress the *det-1* phenotype.

Candidate Gene Analysis

Results of both mapping experiments indicated the region between SSLP makers FCA6.7 and FCA7D2 contained the gene or genes responsible for both the long hypocotyl QTL and suppression of the *det1-1* phenotype. Of the 38 genes in the interval between SSLP markers FCA6.7 and FCA7D2 (Table 3.6), the *HAT4* gene itself (At4g16780) was considered to be the only readily obvious candidate gene for the long hypocotyl QTL/*TED1* (although the region also contains several predicted genes of unknown function). *HAT4* is a class II homeobox-domain (HD-Zip) containing protein involved in the negative transcriptional regulation of genes involved in the shade-avoidance responses under low red to far-red light ratios (R/FR) (Carabelli *et al*., 1993; Schena *et al*., 1993; Steindler *et al*., 1999; Ohgishi *et al*., 2001). In studies where the overall expression of *HAT4* was increased or decreased, hypocotyl length increased or decreased

Table 3.8. Single marker analysis for F_2(TAM826)A.

Chromosome	Marker	b_0	b_1	$-2\ln(L_0/L_1)$	F(1,n-2)	pr(F)	Level[a]
1	PHYA	3.763	-1.019	0.225	0.215	0.645	
1	nga392	3.763	-0.479	0.225	0.215	0.645	
1	nga280	3.763	-0.149	0.225	0.215	0.645	
1	nga111	3.763	-0.336	0.225	0.215	0.645	
2	PHYB	3.743	0.290	0.230	0.220	0.641	
2	nga1126	3.743	0.199	0.230	0.220	0.641	
2	COP1	3.817	-0.303	0.514	0.494	0.486	
2	nga168	3.860	-0.412	0.948	0.914	0.344	
3	nga162	3.677	-1.094	12.765	14.136	0.001	***
3	AtGAPAB	3.669	-1.068	8.932	9.453	0.004	**
3	CIW4	3.760	-0.056	0.027	0.026	0.873	
3	FUS6	3.753	-0.104	0.070	0.067	0.797	
3	nga6	3.750	-0.117	0.094	0.090	0.766	
4	nga8	4.308	0.556	0.122	0.117	0.734	
4	DET1	3.766	0.000	0.000	0.000	1.000	
4	COP9	4.723	1.471	8.991	9.523	0.004	**
4	SC5	4.542	1.419	11.825	12.950	0.001	***
4	FCA6.7	4.556	1.444	11.342	12.350	0.001	***
4	HAT4	4.398	1.157	6.400	6.576	0.014	****
4	FCA7D2	4.087	0.589	2.126	2.079	0.157	*
4	T6K21	4.150	0.998	9.003	9.536	0.004	**
4	nga1139	3.848	0.453	1.557	1.512	0.226	
4	nga1107	3.817	0.377	1.230	1.190	0.281	
5	HY5	3.768	0.013	0.001	0.001	0.973	
5	nga225	3.721	-0.381	1.326	1.285	0.263	
5	nga106	3.751	-0.162	0.272	0.260	0.613	
5	nga139	3.787	0.103	0.108	0.103	0.750	
5	PHYC	3.769	0.585	1.738	1.692	0.200	
5	CIW9	3.673	0.625	3.168	3.136	0.084	
5	CIW10	3.752	0.498	2.355	2.309	0.136	

[a] Significance level – (*) 0.05, (**) 0.01, (***) 0.001, (****) 0.0001

respectively (Carabelli *et al*., 1993; Schena *et al*., 1993; Smith, 1995; Steindler *et al*.,1997).

The *HAT4* gene was directly sequenced from single seed descent lines Be-0-1-01 and Be-0-1-2-8 as well as Col-0-7. A 2.0 Kbp fragment containing 5' upstream sequences (including a *HAT4* self-regulatory element) was sequenced from single seed descent lines Be-0-1-01 and Be-1-2-8. When possible, both strands of DNA were sequenced from Be-0-1-01 and Be-1-2-8 multiple times to confirm polymorphisms and exclude sequencing errors. A single pass sequencing of the *HAT4* gene from Col-0-7 was compared to sequences downloaded from the NCBI nucleotide database (http://www.ncbi.nlm.nih.gov/) to confirm that Col-0-7 used for sequencing and phenotypic analysis was indeed identical by descent to the Col-0 and Col-4 ecotypes previously sequenced (Arabidopsis Genome Initiative, 2000) of the Col-0 ecotype.

Although there were several single nucleotide polymorphisms (SNPs) between Col-0 and each of the Be-0 single seed descent lines (Figure 3.5), none of them led to amino acid changes in the homeodomain (HD) responsible for DNA binding or the leucine zipper (Zip) domain responsible for protein dimerization. There were also no SNPs in the *HAT4 cis*-acting element responsible for autoregulation of *HAT4* gene expression (Figure 3.6).

Sequencing revealed an abundance of SNPs in or near the borders of introns of the *HAT4* gene (Figure 3.5). To determine if an intron splicing defect could lead to the phenotypic variation seen in the mapping populations, intron-spanning primers were used to amplify fragments from cDNA samples (Figure 3.7). If unspliced or incorrectly

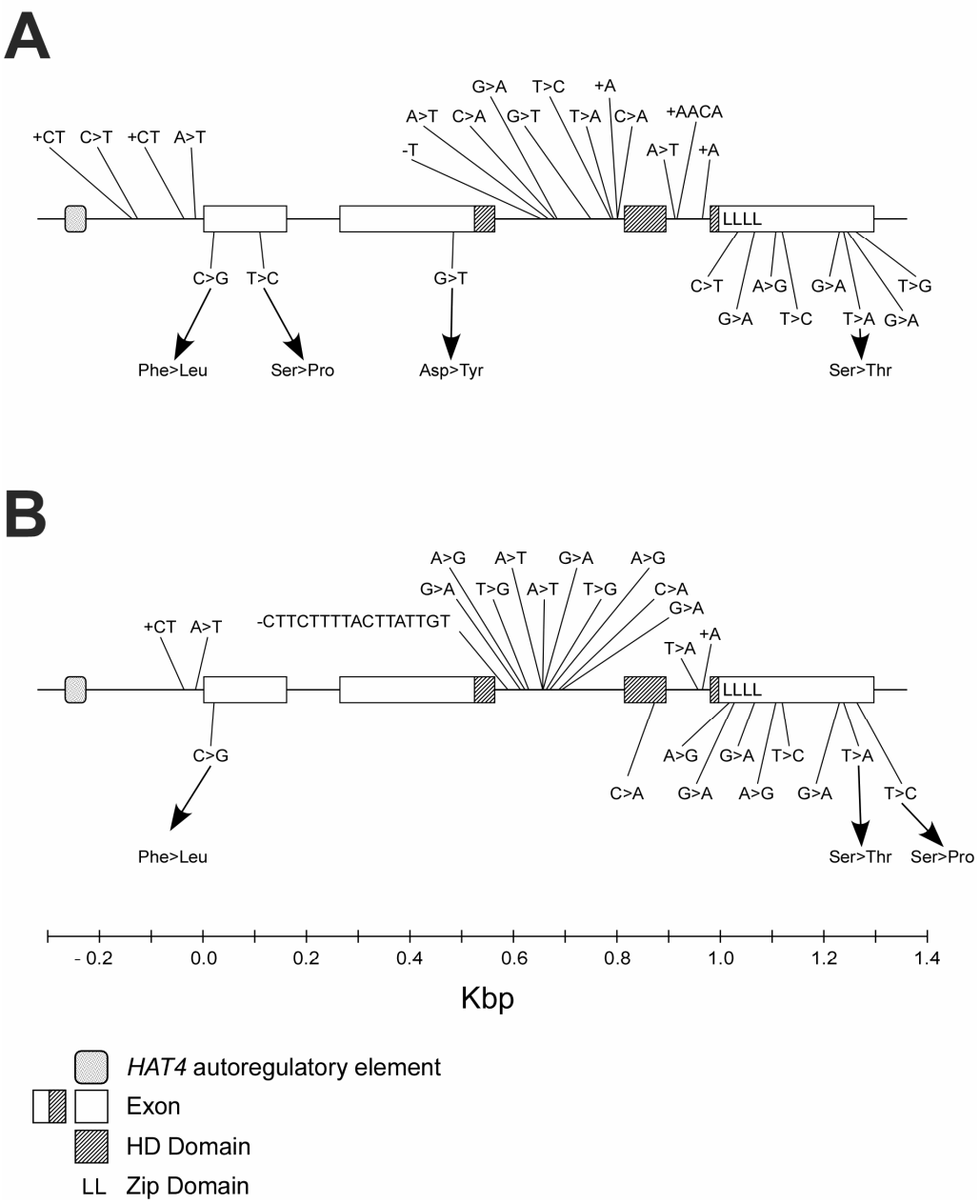


Figure 3.5. Sequence polymorphisms in *HAT4* gene and 5' regulatory sequences.

(A) Polymorphisms between Be-0-1-01 single-seed descent line and Col-0.
(B) Polymorphisms between Be-0-1-2-8 single-seed descent line and Col-0.

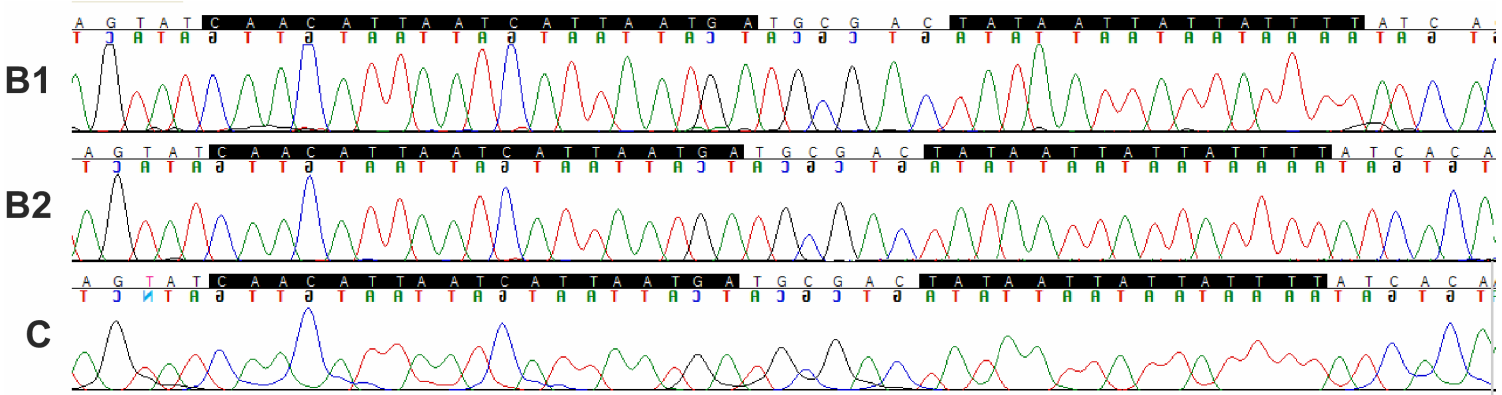


Figure 3.6. Sequence chromatograms from *HAT4* regulatory element.

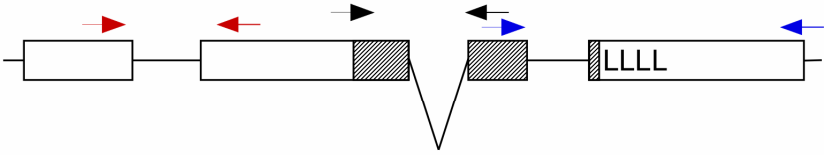
Sequencing chromatograms from the single-seed descent lines Be-0-1-01 (B1), Be-0-1-2-8 (B2) and Col-0-7 (C) showing sequence conservation in the *HAT4* autoregulatory element (highlighted bases).

A

Primer	Sequence (5' > 3')	Position[a]	Intron[b]	Genomic[c]	PCR[d]
HAT4EX1-F	AAGATCTTCATGGAACGAGAGTTTTAC	+126	102 bp	226 bp	124 bp
HAT4EX2-R	GCCGTATTCCGCTGTAGACG	+352			
HAT4EX2B-F	GTGACGATGAAGATGGTGATAACTCC	+459	251 bp	380 bp	129 bp
HAT4E3-R	GCTCGTAACCCTAATTGTTTAGCC	+839			
HAT4E3-F	AATTAGGGTTACGAGCAAGACAAG	+854	87 bp	460 bp	373 bp
HAT4RT-R	GGTTGCAAGTAAAAAGACTTAGGACCTAGGAC	+1314			

[a]Relative to the start of translation
[b]Intron size
[c]Predicted PCR fragment size from genomic DNA
[d]Predicted PCR fragment size from amplified cDNA

B



C

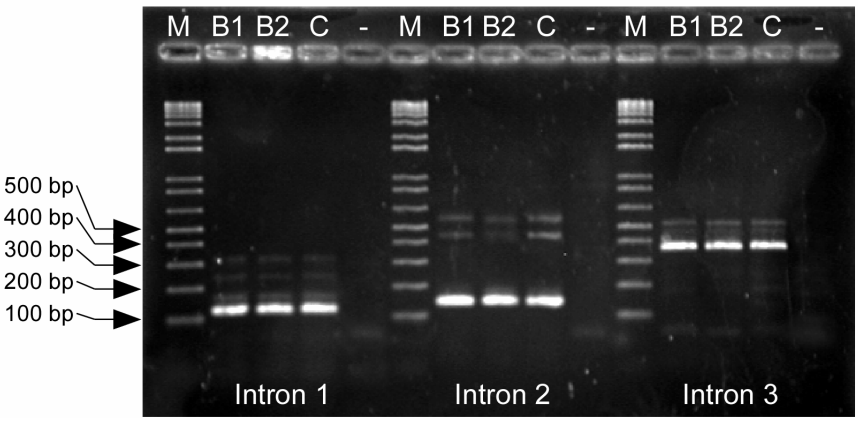
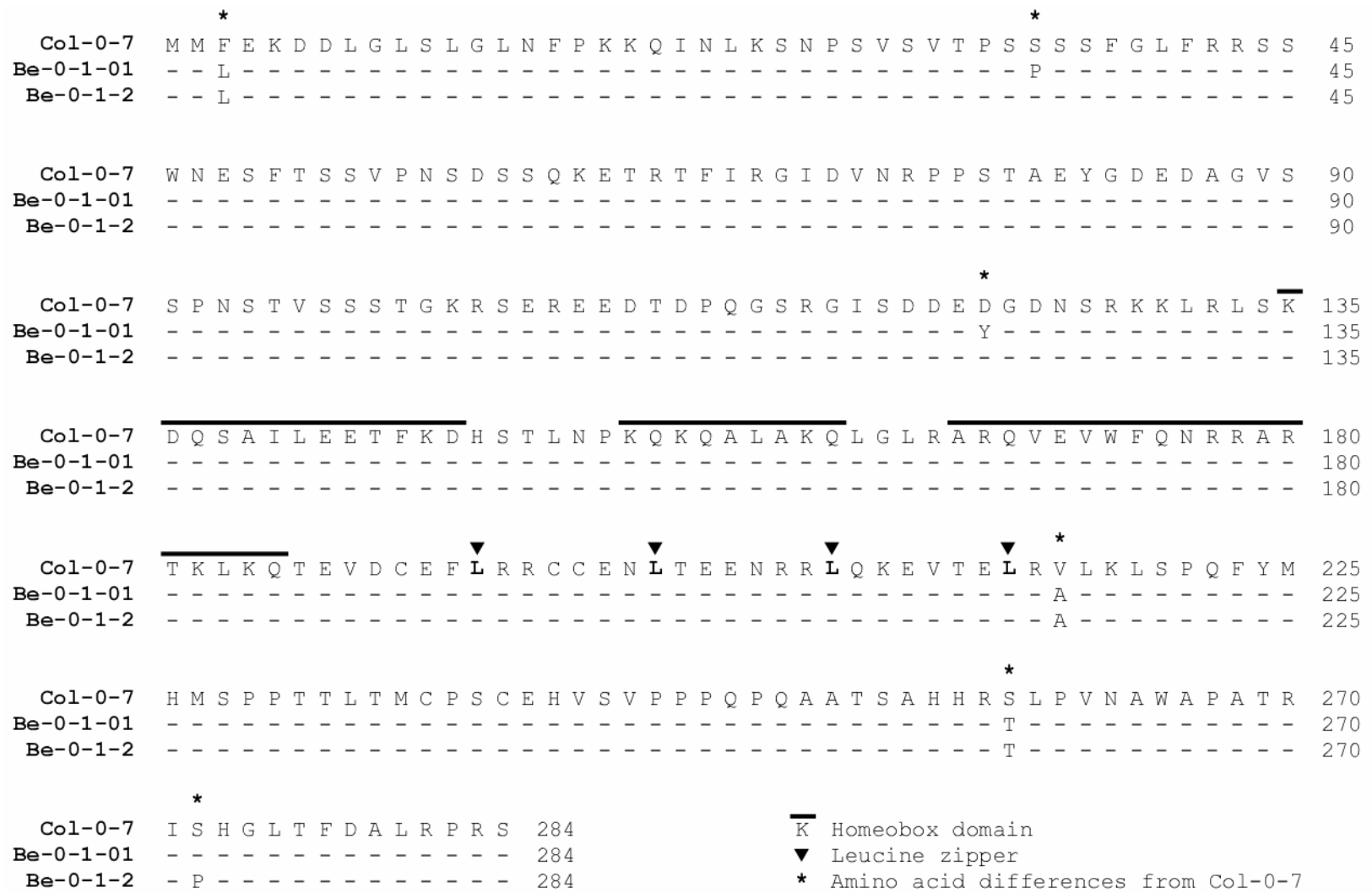


Figure 3.7. Intron splicing evaluation.

(A) Intron spanning primers were used to amplify PCR fragments from cDNA of light growth Be-0-1-01 (B1), Be-0-1-2-8 (B2), and Col-0-7 (C) individuals.
(B) Schematic representation of primer positions in *HAT4* in relation to exons (boxes) and introns (lines).
(C) PCR fragments amplified from cDNA run on a 1% agarose gel with 1 Kb PLUS DNA marker (M). Intron spanning primers amplify identical product sizes in Be-0-1-01 (B1), Be-0-1-2-8 (B2) and Col-0-7 (C) individuals.

Figure 3.8. *HAT4* alignment between Col-0 wild-type and Be-0 single-seed descent lines.

Protein sequence alignment for *HAT4* gene. Homeobox (HD) domain is indicated by a line above the amino acids in the HD domain. Leucine zipper (Zip) domain is indicated by inverted triangles above the leucine residues in the Zip domain. Amino acid differences between Col-0 and either or both of the Be-0 single-seed descent lines are indicated by an asterisk (*)


Col-0-7 M M F E K D D L G L S L G L N F P K K Q I N L K S N P S V S V T P S S S S F G L F R R S S 45
Be-0-1-01 - - L - P - - - - - - - - - - 45
Be-0-1-2 - - L - 45
Col-0-7 W N E S F T S S V P N S D S S Q K E T R T F I R G I D V N R P P S T A E Y G D E D A G V S 90
Be-0-1-01 - 90
Be-0-1-2 - 90
Col-0-7 S P N S T V S S S T G K R S E R E E D T D P Q G S R G I S D D E D G D N S R K K L R L S K 135
Be-0-1-01 - Y - - - - - - - - - - - - - 135
Be-0-1-2 - 135
Col-0-7 D Q S A I L E E T F K D H S T L N P K Q K Q A L A K Q L G L R A R Q V E V W F Q N R R A R 180
Be-0-1-01 - 180
Be-0-1-2 - 180
Col-0-7 T K L K Q T E V D C E F L R R C C E N L T E E N R R L Q K E V T E L R V L K L S P Q F Y M 225
Be-0-1-01 - A - - - - - - - - - - 225
Be-0-1-2 - A - - - - - - - - - - 225
Col-0-7 H M S P P T T L T M C P S C E H V S V P P P Q P Q A A T S A H H R S L P V N A W A P A T R 270
Be-0-1-01 - T - - - - - - - - - - 270
Be-0-1-2 - T - - - - - - - - - - 270
Col-0-7 I S H G L T F D A L R P R S 284
Be-0-1-01 - - - - - - - - - - - - - - 284
Be-0-1-2 - P - - - - - - - - - - - - - 284
K Homeobox domain
▼ Leucine zipper
* Amino acid differences from Col-0-7

spliced introns were present, then the PCR amplification products would be the same size as those amplified from genomic DNA. Intron splicing defects were not observed in the Be-0 single seed descent lines when compared to Col-0. Therefore, any functional variation in the *HAT4* alleles in Be-0 is not due to defective splicing caused by an unspliced intron.

Most SNPs in exon coding regions between Col-0 and the Be-0 lines were synonymous or led to conservative amino acid substitutions (Figure 3.8). Two SNPs causing amino acid substitutions most likely leading to a functional change of *HAT4* were Ser(35) to Pro(35) and Ser(272) to Pro(272) substitutions in the Be-0-1-01 and Be-0-1-2-8 respectively (Figure 3.8). These substitutions would most likely lead to a bend or "kink" in the protein due to the proline substitution. The SNP leading to the Ser(272) to Pro(272) substitution in the Be-0-1-2-8 line also introduces an EcoRV CAPS marker that can be used to confirm the sequence data and as marker for mapping. The Be-0-1-01 allele also contains a SNP (C > T) 801 bp from the translational start site (in intron 2) creating a distinct EcoRV CAPS marker (Figure 3.9). The CAPS marker in the Be-0-1-01 line was also used as a marker for mapping the long hypocotyl QTL and the putative suppressor of *det1-1* Be-0 background.

Genetic effects on phenotype under different light environments

Seedlings of single-seed descent Be-0 lines containing both functionally distinct alleles of *HAT4* as well as Col-0 and *det1-1* allele were grown under a variety of light environments to compare their photomorphogenic responses. Approximately 60

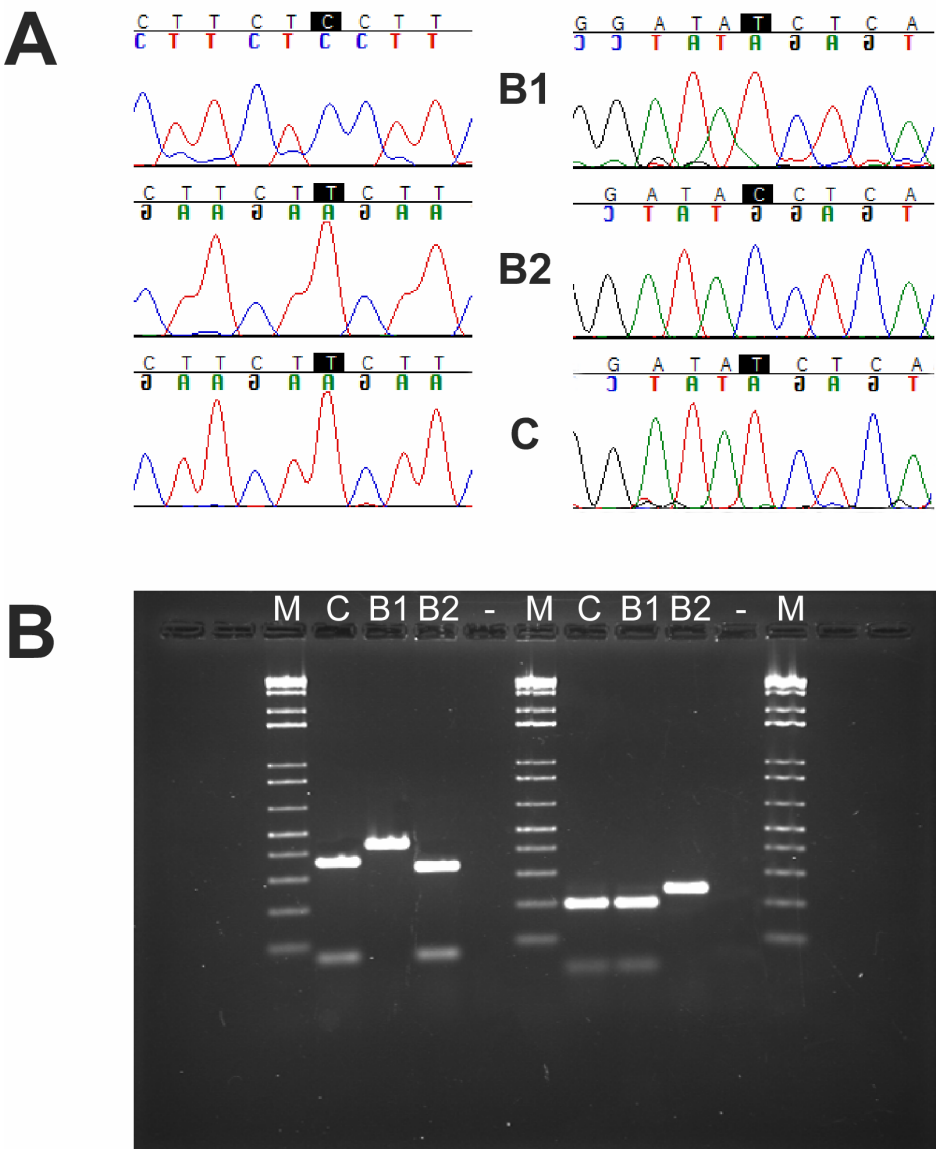


Figure 3.9. Functionally significant SNPs in natural alleles of *HAT4*.

(A) Sequencing chromatograms showing SNPs leading to Serine to Proline amino acid substitutions in Be-0-1-01 (B1) and Be-0-1-2-8 (B2) when compared to Col-0-7 (C). Neither allele contains the substitution in the HD-Zip domain.
(B) Allele specific CAPS markers distinguishing between Be-0-1-01 and Be-0-1-2-8 digested with EcoRV and electrophoresed on a 1% agarose gel.

individuals were measured for each of the ecotypes Col-0-7, Be-0-1-01, and Be-0-1-2-8 to determine average hypocotyl lengths under each light environment. Fewer seedlings for the Be-0-1-2-8 line (approximately 25) were measured under each light condition due to germination deficiencies. Photographs were taken of representative individuals from each ecotype line (Figure 3.10). Additionally, imprints of hypocotyls from each of the genetic backgrounds were made in order to compare epidermal cell dimensions under the medium white light condition (Figure 3.11). A sibling single-seed descent line, Be-0-1-2-36, was used in place of Be-0-1-2-8 for agarose imprints due to seed availability and viability.

Based on hypocotyl length data (which is a quantitative trait commonly used to measure photomorphogenic responses), the Be-0 genetic backgrounds containing functionally distinct alleles of *HAT4* are similar in morphology to the Col-0 wild-type in the dark, but have altered levels of sensitivity in different light environments. The Be-0-1-2-8 genetic background is significantly more sensitive (having a shorter hypocotyl) to red light than the Be-0-1-01 and Col-0. Under far-red light both Be-0 genetic backgrounds are more sensitive than Col-0, but Be-0-1-2-8 is intermediate between Col-0 and Be-0-1-01 in response to far-red light. Interestingly, both Be-0 genetic backgrounds have less sensitivity to blue light than Col-0. Under the medium white light condition (which is a mixture of the other light conditions examined), both Be-0-1-01 and Be-0-1-2-8 genetic backgrounds are less sensitive than Col-0-7 (taller than Col-0-7). Hypocotyl cell measurements from agarose impressions also show that differences in hypocotyl length can be attributed to differences in cell elongation versus number of cell

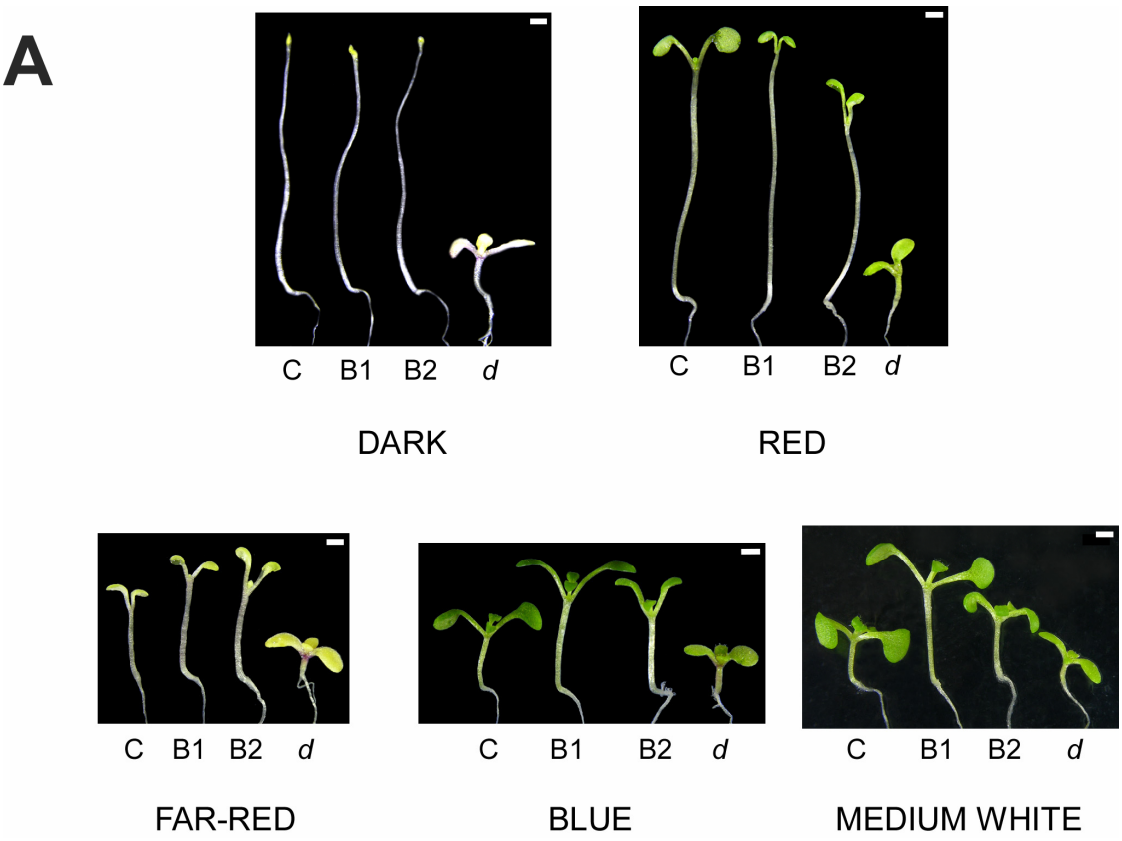


Figure 3.10. Genetic effect on phenotype under different environments.

Seeds from single-seed descent lines Col-0-7 (C), Be-0-1-01 (B1), and Be-0-1-2-8 (B2) were grown under different light environments along with seeds from a pooled sample of *det1-1* mutants.
(A) Photographs of representative individuals of each genotype under the different light environments examined (bar = 1 mm).

B

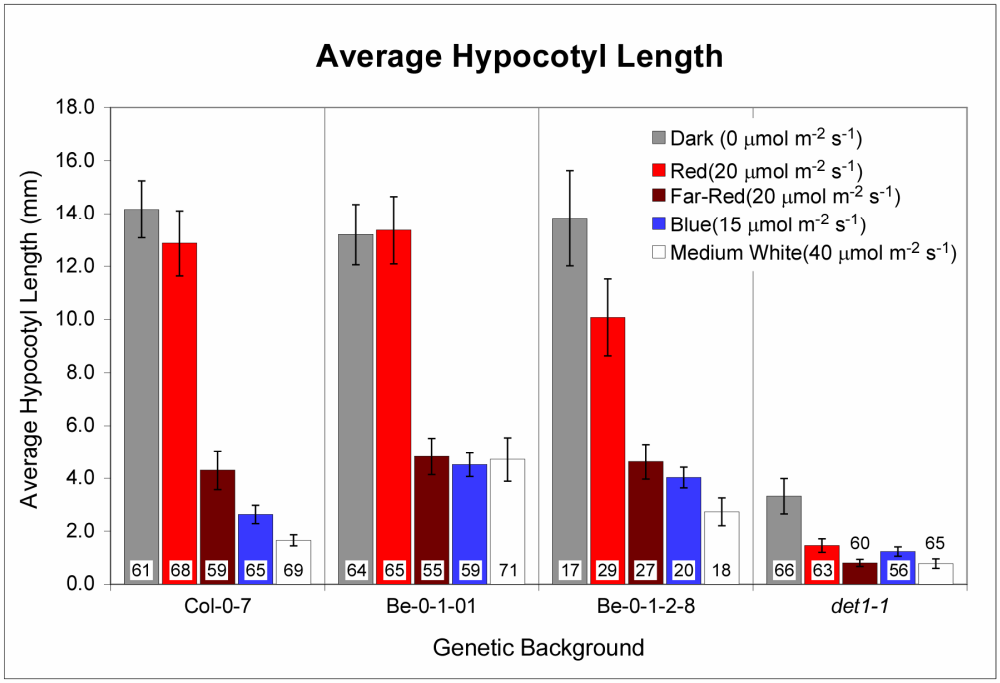


Figure 3.10. Continued.

(B) Average hypocotyl lengths of each genotype under the different light environments examined. Error bars represent standard deviation for average hypocotyl lengths. Numbers represent individuals measured for each light condition.

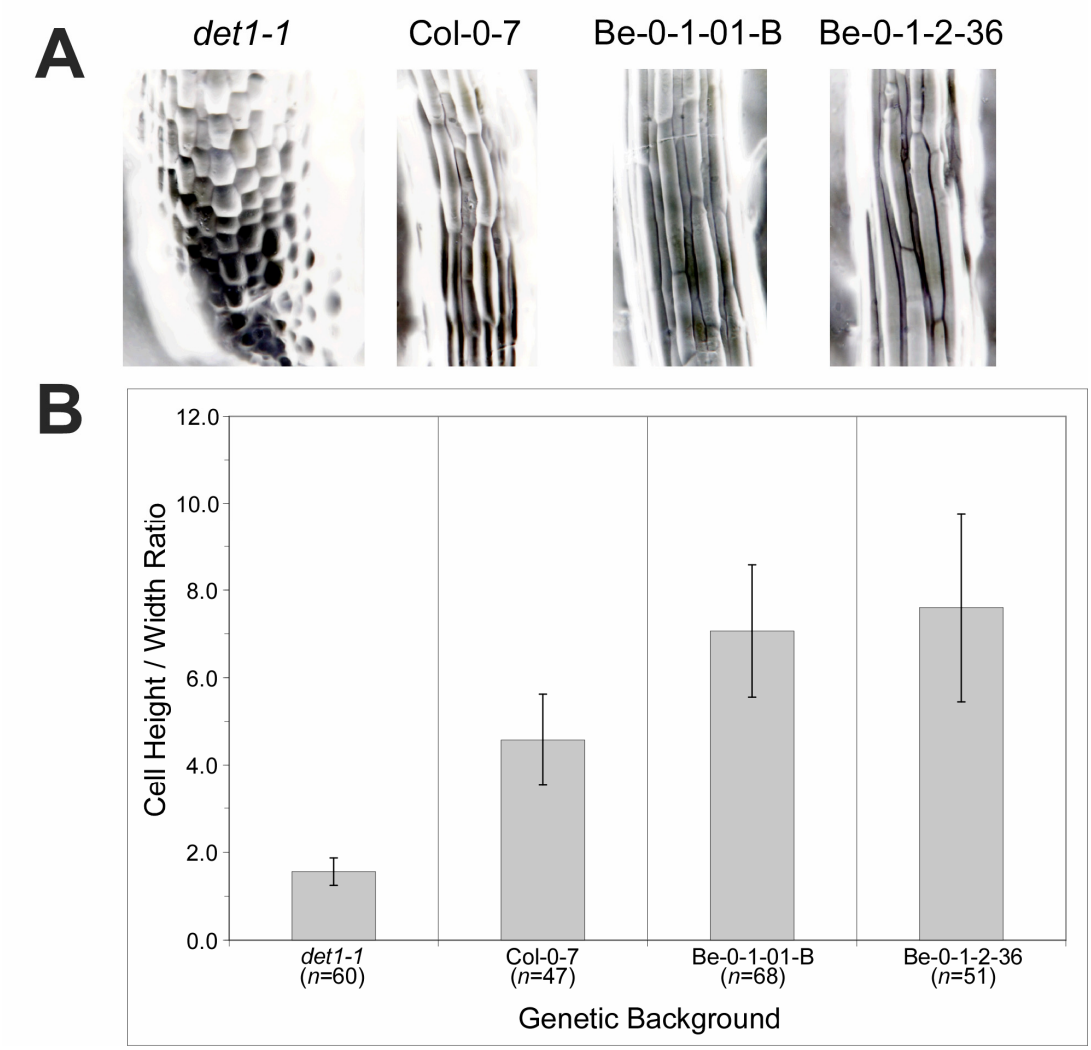


Figure 3.11. Hypocotyl cell analysis.

Seedlings from genetic backgrounds were grown under medium white light (40 μmol m^{-2} s^{-1}) for 8 days. Epidermal cells were examined after impressions were made with molten agarose.
(A) Photographs of agarose impression from seedlings grown under medium white light.
(B) Average cell height to width ratio for genetic backgrounds examined using agarose imprints. Error bars equal standard deviations.

divisions (Figure 3.11). Hypocotyl and cell measurements support the idea that differences in the *HAT4* gene effect hypocotyl length by directly affecting the length of hypocotyl cells.

Analysis of HAT4 and DET1 expression using quantitative RT-PCR

To aid in the development of a model for the suppression of the *det1-1* phenotype and the mechanism of the long hypocotyl QTL, the expression levels of *HAT4* and *DET1* were analyzed under a medium light condition (40 μmol m^{-2} s^{-1}) which confers the greatest phenotypic discrimination between Col-0 and Be-0 (Table 3.9). Additional quantitative RT-PCR experiments were performed in narrow spectrum red light (20 μmol m^{-2} s^{-1}), narrow spectrum far-red light (20 μmol m^{-2} s^{-1}), and the dark. Genotypes examined were single seed descent lines Col-0-7, Be-0-1-01, Be-0-1-2-8 and homozygous *det1-1*. RNA was extracted from seedlings grown in a 5 mm grid pattern after 8 days of growth under each of these conditions, then reverse transcribed into cDNA and used as templates for quantitative RT-PCR with gene specific primers for *HAT4*, *DET1*, and the constitutively expressed internal control *EF1α*. Expression levels (ΔC_t) of *HAT4* and *DET1* were calculated by subtracting the C_t for the *EF1α* control for each ecotype line and treatment combination (Figures 3.12 and 3.13). The lower a ΔC_t value for *HAT4* or *DET1* in an ecotype line and light treatment combination indicates a higher expression level for the gene being examined.

Table 3.9. C_t Values for genes analyzed using RT-PCR.

		Col-0-7		*det1-1*		Be-0-1-01		Be-0-1-2-8	
Gene	Treatment[a]	Average C_t[b]	StDev[c]	Average C_t	StDev	Average C_t	StDev	Average C_t	StDev
HAT4	MWL	26.01	0.26	25.92	0.13	25.31	0.15	26.30	0.07
	DK	24.58	0.09	22.63	0.07	23.15	0.13	23.33	0.10
	RL	27.00	0.17	24.97	0.38	25.97	0.20	26.42	0.20
	FRL	25.76	0.25	23.66	0.21	24.39	0.09	23.55	0.26
DET1	MWL	21.41	0.26	21.34	0.25	21.95	0.28	22.54	0.04
	DK	21.22	0.29	20.98	0.04	22.14	0.19	22.47	0.07
	RL	22.51	0.07	22.42	0.18	22.44	0.16	22.81	0.26
	FRL	22.05	0.13	21.80	0.06	22.91	0.11	22.52	0.19
EF1α	MWL	17.01	0.19	17.67	0.06	17.32	0.06	18.45	0.14
	DK	16.63	0.19	17.89	0.05	17.67	0.20	18.44	0.07
	RL	17.58	0.09	18.30	0.12	18.08	0.08	18.37	0.22
	FRL	17.11	0.07	18.13	0.16	18.55	0.23	18.38	0.21

[a] (MWL) Moderate White Light (40 μmol m^{-2} s^{-1}), (DK) Dark, (RL) Red Light enriched (20 μmol m^{-2} s^{-1}), (FRL) Far-Red Light enriched (20 μmol m^{-2} s^{-1})

[b] Average C_t values for 2 to 3 replicates

[c] Standard Deviation for 2 to 3 replicates

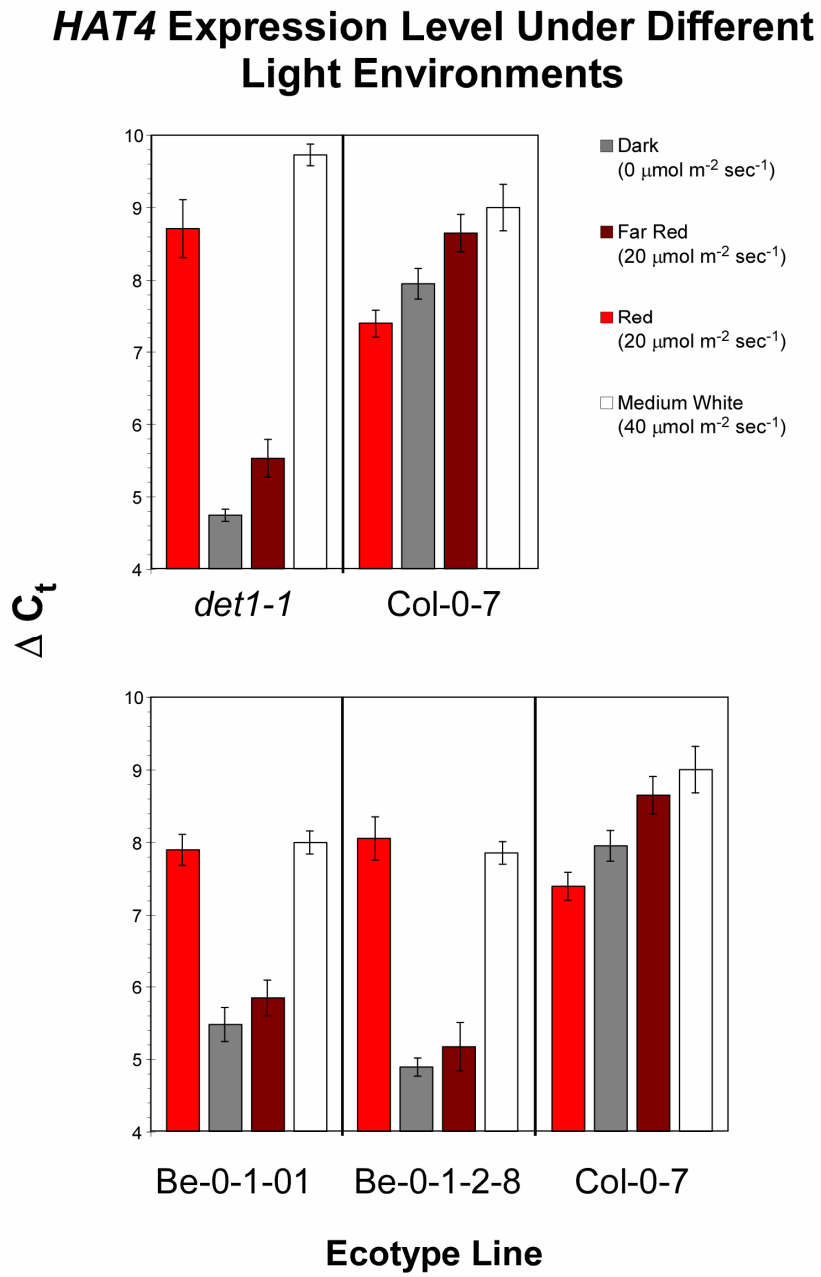


Figure 3.12. Gene expression levels for *HAT4.*

Gene expression levels of *HAT4* in different genetic backgrounds under different light environments. Error bars = standard deviation of ΔC_t value calculated by $(\sigma^2_{gene} + \sigma^2_{control})^{1/2}$.

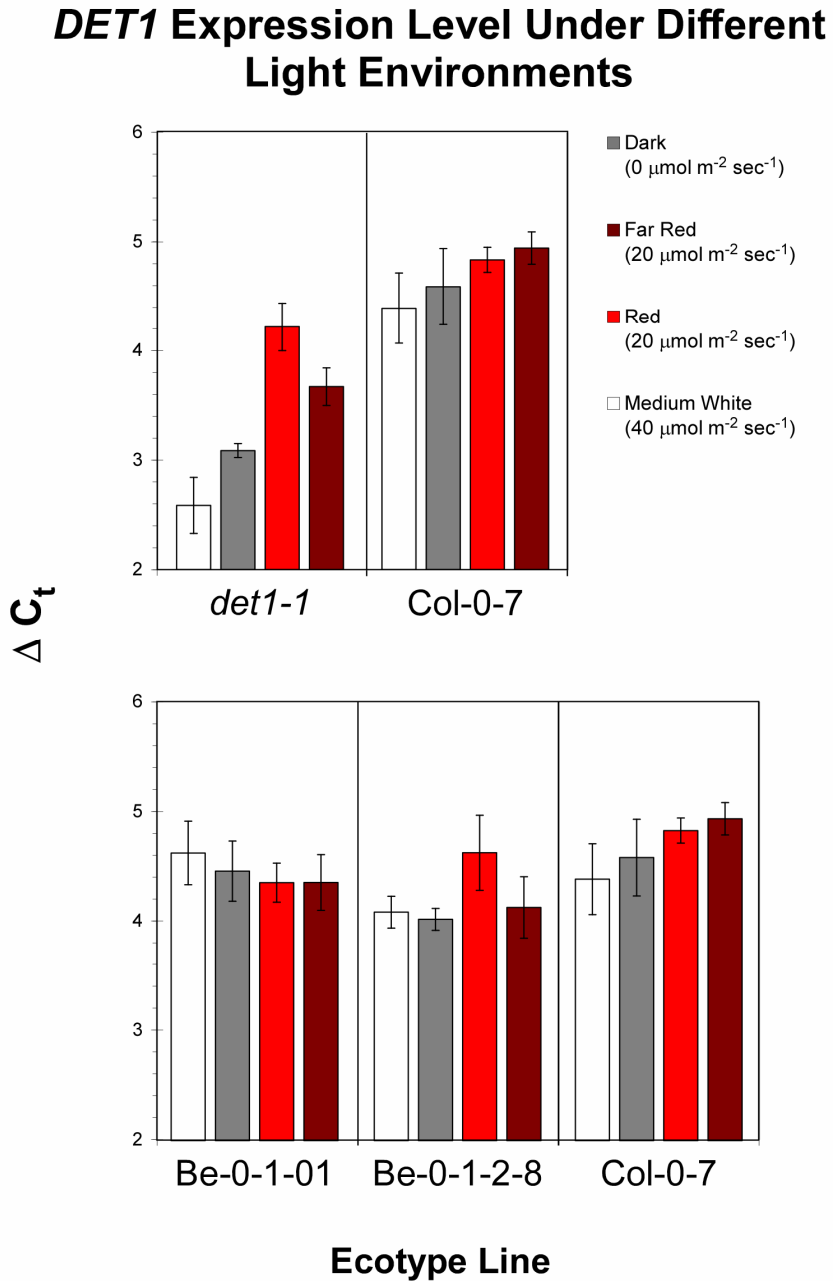


Figure 3.13. Gene expression levels for *DET1*.

Gene expression levels of *DET1* in different genetic backgrounds under different light environments. Error bars = standard deviation of ΔC_t value calculated by $(\sigma^2_{gene} + \sigma^2_{control})^{1/2}$.

DISCUSSION

The long-hypocotyl QTL from the Bensheim (Be-0) ecotype and the *det1-1* suppressor allele from the Be-0 ecotype both mapped – with high statistical significance – to the same region of the Arabidopsis genome. Based on these data, we concluded that the long-hypocotyl QTL and the *det1-1* suppressor are determined by the natural allelic variation at a single locus in the interval between FCA6.7 and FCA7D2. Within this 191 Kbp interval there are 38 genes. Of these genes, the transcription factor *HAT4* stands out as an obvious candidate for this locus.

HAT4 encodes a developmental regulator (Schena and Davis, 1992; Schena *et al*., 1993) that is a mediator of the shade-avoidance responses (SAR) which are triggered by low red to far-red light conditions (low R/FR) encountered when plants are grown in close proximity to or shaded by larger plants. This response induces rapid elongation of the hypocotyl and petioles to allow the plant to compete with neighboring plants in order to obtain more light. Additionally it has been shown that *HAT4* also mediates an end of day (EOD) far-red response in which plants exposed to low R/FR during twilight hours have phenotypes resembling those seen in the SAR. Plants grown under low R/FR conditions seen in SAR and EOD tend to flower earlier than those grown under higher R/FR conditions (Schena *et al*., 1993; Schena and Davis, 1994).

Furthermore, the long-hypocotyl QTL and the suppressor of *det1-1* present in the Be-0 background show the most statistically-significant linkage (in QTL mapping experiments) to a molecular marker derived from the *HAT4* gene itself. Sequencing of the *HAT4* gene revealed that not only does the Be-0 *HAT4* gene contain amino acid

substitutions that could likely alter the configuration and possibly the function of the protein (Figure 3.2), but that our lab stocks of Be-0 also contain two functionally distinct alleles of *HAT4* that may give rise to altered phenotypes under different environments. These findings reveal a previously unknown genetic (and possibly molecular) interaction between *DET1* and *HAT4*. Further, these data suggest that *HAT4* may be an important locus underlying variation in photomorphogenetic responses in natural populations of Arabidopsis.

Sequence Analysis of *HAT4*

HAT4 belongs to the homeobox domain-leucine zipper (HD-Zip) II class of transcription factors that is unique to plants. The HD-Zip II class of transcription factors is a multigene family containing proteins with a homeobox domain (HD) in close proximity to a leucine zipper domain (Zip) unlike the homeobox domain transcription regulators found in animals which lack an adjacent leucine zipper domain.

The fact that some HD-Zip proteins have very similar DNA binding specificities but regulate distinct biological pathways has led to the hypothesis that targeting of HD-Zip proteins *in vivo* to the correct promoters depends on interactions with other proteins (Johannesson, 2000). While other HD-Zip proteins are thought to be able to form heterodimers in order to regulate different sets of genes, it has been shown that *HAT4* exclusively forms homodimers and binds to a 9 bp pseudopalindromic sequence (CAAT(G/C)ATTG) via helix 3 of the homeodomain in the major groove of the DNA (Sessa *et al*., 1993, 1997). In addition to being a transcriptional regulator for genes

involved in the SAR and EOD pathways, *HAT4* also regulates its own expression by a negative autoregulatory feedback loop via binding to its own promoter at a *cis*-acting regulatory element 240 bp upstream of the transcriptional start site (Ohgishi *et al*., 2001). Mutations in either the binding site for *HAT4* or in the homeodomain have been shown to either diminish or completely abolish binding of *HAT4* to DNA (Sessa *et al*., 1997). Neither of the functionally distinct alleles of *HAT4* from the Be-0 ecotype lines in our lab contain mutations that would effect the amino acid sequence of helix 3 of the homeodomain necessary for DNA binding or change the sequence of the binding site necessary for autoregulation of *HAT4* expression (Figures 3.5 and 3.8).

The amino terminus of HD-Zip proteins (5' to the HD) has been shown to interact with DNA in the minor groove adjacent to the HD binding site in the major groove, and amino acids positioned in the loop between helix 1 and 2 and at the start of helix 2 make contacts with the DNA backbone itself (Johannesson, 2000). These interactions along with the dimerization of HD-Zip proteins before binding to DNA is thought to stabilize the protein / DNA complex since binding in two adjacent major grooves of the DNA helix imparts strain on the DNA helix itself. The carboxyl terminus (3' to the Zip) is available for interactions with other proteins that could be possibly involved in regulation of transcription (Johannesson, 2000).

The Be-0-1-01 allele of *HAT4* contains a Ser(35) to Pro(35) substitution in the amino terminus of the protein. The Be-0-1-2-8 allele of *HAT4* contains a Ser(272) to Pro(272) in the carboxy terminus of the protein, but not the Ser(35) to Pro(35) substitution in the amino terminus of the protein seen in the Be-0-1-01 allele. The Be-0-

1-01 allele most likely destabilizes the interaction of the amino terminus with the minor groove of the DNA due to the substitution of a proline residue for a serine residue which would cause a 'kink' in amino terminus. The Be-0-1-2-8 allele could possibly interfere with dimerization of the *HAT4* monomers and/or binding stability of the *HAT4* dimer as well as interactions of *HAT4* with other transcription factors. Both alleles likely effect binding stability of *HAT4* to regulatory sequences effecting transcriptional regulation. This binding destabilization could explain why *HAT4* is expressed at higher levels in the Be-0 allelic lines when compared to Col-0, as the negative autoregulation would be less effective due to lower binding of *HAT4* to the regulatory sequence in its own promoter.

Our sequence analysis of the Be-0 alleles of *HAT4* (in comparison to Col-0) revealed an unexpected level of functionally- and phenotypically-significant diversity. We were surprised to find two functionally distinct alleles of *HAT4* (heterozygosity) within a single-seed descent Be-0 line. These findings suggest that there may be high levels of functional genetic variation at the *HAT4* locus within natural populations of Arabidopsis and this diversity may underlie much of the phenotypic diversity in photomorphogenic responses in natural populations (Maloof *et al*., 2001; Botto and Smith, 2002; Pepper *et al*., 2002)

Expression Analysis

In wild-type Col-0 plants, *HAT4* expression is elevated in dark grown plants, but is quickly down regulated upon exposure to white light, red light, or far-red light (Carabelli *et al*., 1993; Carabelli *et al*., 1996). For light grown plants, exposure to low

R/FR light levels induces a rapid increase in *HAT4* expression levels which can be reversed by a higher R/FR light level (Carabelli *et al*., 1993). Exposure of etiolated seedlings to either red or far-red light, reduces the level of *HAT4* expression.

Our quantitative RT-PCR analysis uncovers a functional relationship between *DET1* and *HAT4*. When comparing expression levels of *HAT4* in the *det1-1* mutant (in the Col-0 genetic background) to those seen in wild-type Col-0, *HAT4* is expressed at higher levels under dark and far-red light conditions. The difference between the *det1-1* mutant and Col-0 is the mutation in the *DET1* gene which causes a splicing defect in the mutant such that the first intron remains unspliced leading to an early termination during translation with a small fraction (approximately 2%) of the mRNA spliced correctly and translated into a functional protein (Pepper and Chory, 1997). These results supply evidence that *HAT4* is negatively regulated by *DET1* at the transcriptional level. The elevated levels of *HAT4* expression in Be-0 allelic backgrounds in dark, far-red light, and medium white light, compared to the Col-0 background, are either due to the polymorphisms in the *HAT4* gene itself (that effect autoregulation) or the action of another gene(s) which differs between the Col-0 and Be-0 genetic backgrounds (possibly including *DET1*).

Quantitative RT-PCR revealed that *DET1* was expressed at similar levels in the dark and the light in the wild-type control Col-0-7 (Figure 3.12). *DET1* expression varies only slightly (Δ C_t difference ≤ 1 equivalent to less than 2-fold difference) under all light conditions for both Be-0 alleles when compared to Col-0, but under some light conditions *DET1* is expressed at a slightly higher level in the Be-0 alleles. The Be-0-1-2-

8 line has a higher expression level of *DET1* compared to the Be-0-1-01 line under far-red light which correlates with the fact that the Be-0-1-2-8 allele of *HAT4* is expressed at a higher level under far-red light than the Be-0-1-01 allele of *HAT4*. Probably the most revealing data from the quantitative RT-PCR experiment was the fact that the *det1-1* mutant has the highest level of *DET1* expression in the light of all genetic backgrounds examined which is most likely due to stabilization of the mis-spliced mRNA which has been shown previously (Pepper *et al.*, 1994).

Models for Suppression of *det1-1* by Bensheim Allele of *HAT4*

The work presented here suggests two non-mutually exclusive models for the suppression of the *det1-1* allele by the Be-0 alleles of *HAT4*. In the first model, DET1 and HAT4 act in an additive manner to regulate hypocotyl growth. The *det1-1* allele causes short hypocotyl growth in both the light and the dark. Both Be-0 alleles of *HAT4* cause long hypocotyl growth in the wild-type background. Hence the additive effects of crossing Be-0 to *det1-1* result in partial suppression of the short hypocotyl in *det1-1*. This model accommodates the possibility that *HAT4* and *DET1* are acting in independent pathways.

The second model is proposed on the finding that the *DET1* transcript is expressed at a slightly elevated level in the Be-0 backgrounds compared to Col-0 under some light conditions. The *det1-1* allele is a partial loss-of-function allele with ~2% of the mRNA transcript correctly spliced. The *det1-1* mutant shows elevated levels of *DET1* transcript compared to Col-0. These levels may be further elevated by the Be-0

background in crosses of *det1-1* to Be-0, leading to suppression of the *det1-1* short hypocotyl phenotype. Based on these findings, the prediction would be that the correctly spliced levels of the *DET1* transcript would be ever elevated in *det1-1* when introgressed into the Be-0 genetic background.

FUTURE PLANS

Further Examination of the Function of the Be-0 Alleles of *HAT4*

To further examine the developmental role of the Be-0 alleles of *HAT4* in light signaling and photomorphogenesis we plan to use a transgenic approach. Previous studies looking at the effects of *HAT4* expression levels on plant development and growth used constructs containing the Col-0 *HAT4* allele in the sense and antisense orientations driven by the 35S promoter to raise or lower *HAT4* expression levels respectively (Schena *et al*., 1993) and point mutations affecting the binding specificity of *HAT4* dimer to its own promoter (Sessa *et al*., 1997). While over expression of the Col-0 wild-type *HAT4* gene driven by the 35S promoter results in some similar phenotypic traits seen in the Be-0 ecotype (longer hypocotyl, longer petioles) (Schena *et al*., 1993; Steindler *et al*., 1999), the endogenous *HAT4* gene, and possibly other genes, are repressed due to the negative autoregulatory binding site in the *HAT4* promoter (Ohgishi *et al*., 2001).

Our plans include using SALK lines (Alonso and Stepanova, 2003; Alonso *et al*., 2003) containing T-DNA inserts in the *HAT4* gene to observe the effect of a *bona-fide* *HAT4* null mutation on phenotypic responses to light signaling. After observing the

effects of *HAT4* loss of function in the SALK lines, T-DNA inserts will be constructed from the *HAT4* gene and its promoter sequence derived from Col-0 and both of the Be-0 genetic backgrounds examined in this research for transformation into the SALK lines. This experiment will serve two purposes: to check for complementation of the *HAT4* loss of function phenotype by the Col-0 T-DNA insert, and to observe the effect of the two functionally distinct alleles of *HAT4* isolated from the Be-0 genetic background on photomorphogenesis in the absence of the endogenous Col-0 wild-type *HAT4* (which should reproduce the long hypocotyl phenotype).

To confirm that the Be-0 alleles of *HAT4* are responsible for the suppression of the de-etiolated phenotype of the *det1-1* grown in the dark, SALK lines transformed with the Be-0 alleles exhibiting the long hypocotyl phenotype will be crossed to *det1-1*. F_1 plants confirmed heterozygous for the SALK insert using PCR will be allowed to self and suppression of *det1-1* will be analyzed in the F_2 progeny that are homozygous for the *det1-1* mutation. Additionally, the Be-0 derived *HAT4* T-DNA constructs will be transformed into the homozygous *det1-1* genetic background directly and progeny will be screened for suppression of the *det1-1* phenotype. The experiments will rule out the possibility that other gene(s) in the Be-0 genetic background are responsible for the long hypocotyl phenotype and the suppression of *det1-1*.

Further Examination of the Genetic Diversity at the *HAT4* Locus in Natural Populations

Our sequencing results for *HAT4* in the diverse ecotypes Col-0 and Be-0 have already showed that *HAT4* could be an evolutionary significant transcriptional regulator. Arabidopsis ecotypes are spread throughout the world and our examination of photomorphogenic responses (based on hypocotyl length) has already shown that there is great diversity in light responses (Pepper *et al*., 2002). To examine if *HAT4* is a gene contributing to the natural diversity in photomorphogenesis, we plan to sequence the *HAT4* gene in ecotypes with diverse photomorphogenetic responses (compared to Col-0). This will allow us to discern if there is any relationship between diversity at the *HAT4* locus with diversity in responses to different light conditions.

CHAPTER IV

TED2 SUPPRESSOR OF *det1-1*

OVERVIEW

Large-scale genomic methods were developed to assist in refining the position for *ted2*, a dominant extragenic suppressor of the photomorphogenetic mutant *det1-1*. Suppression of the *det1-1* dark grown phenotype mapped to a 57 Kbp region on chromosome I containing three likely candidate genes: 1) an armadillo / β-catenin repeat protein / U-box containing protein, possibly involved in protein degradation via the ubiquitin-proteosome pathway, 2) an auxin efflux carrier protein, and 3) a C2H2 domain containing protein similar to the zinc ring-finger family of transcription factors. Suppression of the *det1-1* light-grown phenotype by *ted2-1D* was discovered to be overdominant which is usually only seen in cases of hybrid vigor. This fact along with sequence analysis of the candidate genes for *TED2* will reveal the identity of *TED2* and the likely mechanism of suppression of the *det1-1* mutation.

INTRODUCTION

In order to further the understanding for the role of *DET1* in photomorphogenesis, suppressor mutations were isolated that partially or fully restored the de-etiolated phenotype of a *det1-1* homozygous mutant grown in the dark to the wild-type etiolated phenotype. The *ted2-1D* mutation was originally isolated in an EMS

mutagenesis screen of *det1-1* homozygous mutant for suppressors of the *det1-1* phenotype. The *det1-1* mutation is a G to A transition located just inside intron 1, five nucleotides from the 5' splice junction leading to a defect in splicing of intron 1(Pepper *et al*., 1994). *ted2-1D* was roughly mapped to a ± 29 cM region between markers nga248 and GAPAB on chromosome I (Pepper and Chory, 1997). The *ted2-1D* mutation suppresses the dark-grown phenotype of *det1-1* in a dominant manner; however it does not restore correct splicing of the *det1-1* transcript. In addition, *ted2-1D* also suppresses or partially suppresses other alleles of *det1* including *det1-4* (a missense mutation) and *det1-6* (a null mutation) (Pepper and Chory, 1997).

MATERIALS AND METHODS

The F_2(TAM754)A population: La-er × ted2-1D (*Col-0*)

Previously, Pepper *et al*. showed that the *ted2-1D* EMS mutation suppressed the dark-grown phenotype of the *det1-1* mutant (Pepper *et al*., 2002). To further fine map this suppressor locus, a large mapping population was created by crossing La-*er* (*TED2/TED2 det1-1/det1-1,* male parent) to *ted2-1D/- det1-1/det1-1* in the Columbia background (female parent) (Table 4.1). The hybrid genotype of F_1 progeny was confirmed by testing for heterozygosity at the *det1* locus using a Cleaved Amplified Polymorphic Sequence (CAPS) marker (Glazebrook *et al*., 1998) specific for the *det1-1* mutation (Pepper and Chory, 1997). Any individuals that were derived from self-pollination of *det1-1* were excluded from further analysis. For this marker, a 117 bp fragment containing the *det1-1* mutation was amplified using primers 13-1G (CTTCGA

ACGTCAGATTCGAACTCCTC) and 13-1revB (CATTGAAGGTAAAGAGATAAG C), then tested for cleavage by the restriction endonuclease *Bsm*FI. The PCR fragment amplified from an individual carrying *det1-1* allele will be cut by *Bsm*FI while an individual carrying the *DET1* allele will not. F_1 seedlings heterozygous for the *det1-1* mutation were allowed to self-pollinate to produce an F_2 mapping population which was then screened using the *det1-1* mutation specific CAPS marker. Since the trait being measured in this population was suppression of the *det1-1* phenotype, only those individuals that were homozygous for the *det1-1* mutation were included in the phenotypic and genotypic analysis.

Table 4.1. Cross used for mapping *ted2-1D* suppressor of *det1-1*

TAMU Registry Number	Male Parent	Female Parent
F_2(TAM754)A	La-*er* (*TED2/TED2 DET1/DET1*)	52LH-H-2 (Col-0) (*ted2-1D/- det1-1 / det1-1*)

Hypocotyl Measurements

Hypocotyl length is a well established experimental trait for the quantification of photomorphogenetic development (Pepper and Chory, 1997; Maloof *et al*., 2001; Botto and Smith, 2002; Pepper *et al*., 2002; Chen *et al*., 2004). Seeds for each mapping population, along with the parents of the F_1 (the parental controls), were sterilized as described in Chory, *et al*. (1989). Seeds were placed in 1.5 ml Eppendorf tubes and soaked in pure ethanol for one minute. Afterwards, the ethanol was removed by

aspiration and the seeds were then soaked in a 2:1, sterile water to bleach solution containing the surfactant Tween 20 for ten minutes. Following bleach treatment, seeds were washed three to four times with sterile water and resuspended in a 0.1% phytagar solution. Seeds were cold treated by incubation at 4 °C for three to four days before plating on growth medium. Growth medium for the hypocotyl measurements was prepared by dispersing 35 mL of MS-2% medium (1 X Murashige and Skoog salts, 1 X Gamborg vitamins, 2% sucrose w/v, 0.8% phytagar w/v) (Murashige and Skoog, 1962; Gamborg *et al*., 1968) into 25 mm X 110 mm polystyrene Petri dishes. Plates were prepared one to two weeks before use in order to check for contamination of the medium before growing seeds.

Seeds were dispersed on the growth medium in a 5 mm grid pattern to ensure even spacing and exposed to four hours of white light (100 μmol m^{-2} s^{-1}) prior to dark treatment to initiate maximal germination. Seedlings either grown for eight days in the dark prior to hypocotyl measurements. Plates were rotated and shuffled under the light sources on a daily basis in order to reduce micro-environmental effects on phenotype within and between plates.

To map the putative *ted2-1D* suppressor of *det1-1*, F_2(TAM754)A seedlings were grown under dark conditions in order to observe the suppression of the *det1-1* dark phenotype. Because a large proportion of seedlings will not recover after seven days in the dark, callus culture was used to generate adequate quantities of tissue for DNA genotyping. After measuring, F_2(TAM754)A seedlings were transferred to numbered positions on a callus induction medium (CIM) (1 X Murashige and Skoog salts, 1 X

Gamborg vitamins, 3% sucrose w/v, 0.8% phytagar w/v, 1.0 mg / L 6-benzylamino-purine (BAP), 0.1 mg / L 1-naphthaleneacetic acid (NAA) (Patton and Meinke, 1988) to produce callus tissue for DNA isolation.

DNA Extraction Methods

In the early part of this project, DNA was extracted from individuals in mapping populations using a modification of the method described by Pepper and Chory (1997). Individual calli were placed in a 1.5 ml Eppendorf tube and ground with a polypropylene pestle until the tissue was broken down before 0.5 ml of extraction buffer (200 mM Tris pH 7.5, 250 mM NaCl, 25 mM EDTA pH 8.0, 0.5% SDS) was added. After addition of the extraction buffer, samples were further ground until the tissue was broken down completely. Samples were centrifuged for four minutes at 16,000 × g (14,000 rpm) in a tabletop Eppendorf 5415C centrifuge to pellet the cellular debris and 450 µl of the supernatant was transferred to a new 1.5 ml Eppendorf tube containing 450 µl of isopropanol. Tubes were inverted to mix and the DNA was precipitated for ten minutes at room temperature. Following precipitation, samples were centrifuged for four minutes at 16,000 × g to pellet the DNA and the supernatant was decanted off of the resulting pellet. DNA pellets were resuspended in 50 mM Tris pH 7.5, 10 mM EDTA pH 8.0 using another polypropylene pestle and centrifuged an additional four minutes to remove any solids that did not dissolve. Afterwards, the DNA was precipitated a second time by transferring 450 µl of the supernatant into a new Eppendorf tube containing 50 µl 3 M sodium acetate, pH 5.2 and adding 500 µl isopropanol. DNA was precipitated in the

same manner as before and the resulting pellets were washed with 70% ethanol to remove salts (Na, EDTA) that could possibly inhibit downstream processes. DNA pellets were dried in a Savant DNA110 Speed Vac, resuspended in 0.1 X TE (1 mM Tris pH 7.5, 0.1 mM EDTA pH 8.0), and stored at -20 °C until use.

High through-put (HTP) DNA extraction

It became apparent that DNA extraction from large numbers of individuals in our mapping populations was a major rate-limiting step of this project. In order to speed up the mapping process, new methods of DNA extraction were developed. The first method involved using a Black and Decker™ 9.6V cordless drill to grind the samples using the previous method. While this procedure did speed up the process, the time involved was still considerable because of the sample and reagent transfer steps using a single-channel micropipettor.

A 96-well high through-put (HTP) DNA extraction method for *Arabidopsis* was loosely modeled after the "paint shaker" method of Michaels and Amasino (2001). This and similar methods allow simultaneous DNA extractions of up to 96 samples at the same time. In our method, individual or callus cultures were placed in separate tubes of a Costar macro-tube library plate (Costar part #4413). A 1/8" X 1/2" stainless steel bar (Small Parts Inc., Miami Lakes, Florida, part #DWX-02-08-C) and 500 µl of DNA extraction buffer (200 mM Tris pH 7.5, 250 mM NaCl, 25 mM EDTA pH 8.0, 0.5% SDS) was added to each macro tube and the entire plate was sealed with strip caps (Costar part #4418). Tissue was ground for two minutes in a Spex Centriprep

GenoGrinder 2000 set at 1500 strokes per minute. A brief spin at 3250 rpm in a Beckman GS-6KR centrifuge equipped with a PTS-2000 rotor was done in order to remove droplets from the strip-caps before proceeding. Cellular debris were removed by transferring 400 µl of supernatant from each DNA sample into a 96-well, 0.45 µm, melt-blown polypropylene filter plate (Phenix research products, part # F-20005) seated above a 1 ml, 96-well receiving plate (Phenix research products, part # M-0355) containing 400 µl isopropyl alcohol per well. The assembly was then centrifuged in a Beckman GS-6KR centrifuge at 1,920 × g (3,250 rpm). Samples were mixed by pipetting and left a room temperature for ten minutes to precipitate the DNA then centrifuged at 1,920 × g (3,250 rpm) for thirty minutes to pellet the DNA. The supernatant was removed as quickly as possible after centrifugation in order to prevent loosening of the pellet by inverting the plate over a sink and then placing the plate on a stack of clean paper towels to allow for residual liquid to drain off. After ten minutes, plates containing the DNA pellets were dried to completion using a Savant SC210A Speed Vac Plus and then resuspended in 50 to 100 µl 0.1 X TE (50 mM Tris-HCl, pH 7.5; 0.1 mM EDTA, pH 8.0) depending on the amount of starting tissue. The quality and quantity of DNA isolated from the HTP DNA extraction was examined by running 5 µl of each sample on a 1% agarose gel using standard procedures. Samples were treated with 0.1 µg/ml RNase for one hour and stored at -20 °C until use.

Molecular Marker Development and Genetic Mapping

Simple sequence length polymorphism (SSLP) (Konieczny and Ausubel, 1993) and cleaved amplified polymorphic sequence (CAPS) (Bell and Ecker, 1994) marker methods used for mapping of the putative *ted2-1D* suppressor of *det1-1* were developed from polymorphism sequence and information obtained from The Arabidopsis Information Resource (TAIR) website (http://www.arabidopsis.org) or were designed from sequence data generated in our laboratory. Additional SSLP markers were created by using the Monsanto Arabidopsis polymorphism and Landsberg-*erecta* sequence collection available on the TAIR website (Jander *et al*., 2002). Primer pairs were designed that would amplify regions containing these repetitive (and often polymorphic) sequences. A full list of markers created in our lab and their related information can be found in Appendix B. Individuals in the mapping population, along with genomic controls from the parents, were amplified with a set of polymorphic markers using standard PCR conditions (Bell and Ecker, 1994).

PCR fragments amplified from individuals were electrophoresed on 4% agarose gels (2% Metaphor™ agarose, 2% agarose) at 15 V cm^{-1} using a modified 0.5 X TBE (0.088 M Tris-borate, 0.001 M EDTA) as running buffer. Tabletop fans were used to circulate air around running chambers during electrophoresis to prevent overheating. Gels were stained with ethidium bromide after electrophoresis and visualized using an Alpha Innotech gel documentation system.

RESULTS

Mapping the *ted2-1D* Suppressor of *det1-1*

Hypocotyl length distributions

The F_2(TAM754)A population was used to map the *ted2-1D* suppressor of the *det1-1* phenotype in the dark. The frequency distribution of the F_2(TAM754)A population showed a segregation of roughly 3:1 for individuals with hypocotyl length similar to the Col-0 and La-*er* controls to individuals with hypocotyl length similar to the *det1-1* control (Figure 4.1). This segregation is easily explained by the fact that *det1-1* is a recessive mutation. The homozygous *det1-1* individuals showed a wide distribution from individuals with hypocotyls as short as the *det1-1* control to those with hypocotyls as long as the La-*er* control (which is slightly longer than the Col-0 control). In addition to the *ted2-1D* mutation suppressing the *det1-1* short hypocotyl length in the dark, there may also be additional genes affecting hypocotyl length between Col-0 and La-*er* since some of the F_2 individuals are longer than the Col-0 control. Only those individuals homozygous for the *det1-1* mutation were used for mapping (approximately ¼ of the total number of individuals measured) (Table 4.2).

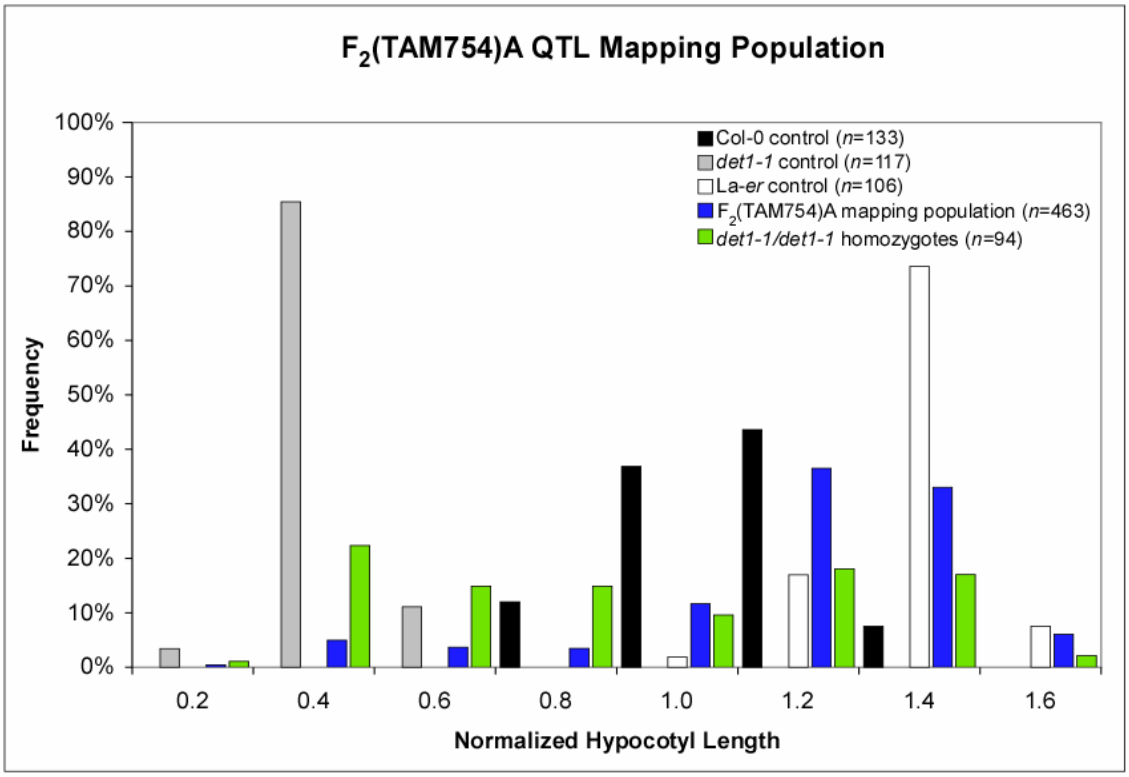


Figure 4.1. Hypocotyl length distributions for F_2(TAM754)A mapping population.

Hypocotyl lengths were normalized on each Petri plate to the average Col-0 to eliminate microenvironmental effects within and between plates on phenotypic quantitative data. Only individuals that were homozygous for the *det1-1* mutation were used for mapping of the *ted2-1D* suppressor of *det1-1*.

Table 4.2. Summary of *det1-1* suppressor in Be-0 mapping populations.

Mapping population	$\mathbf{n_{(measured)}}$	$\mathbf{n_{(det1\text{-}1/det1\text{-}1)}}$	$\mathbf{n_{(genotyped)}}$
F_2(TAM754)A	463	94	92

Fine mapping of ted2-1D suppressor of det1-1

Due to the fact that individuals in the F_2(TAM754)A mapping population were screened for homozygosity of the *det1-1* mutation before mapping of the *ted2-1D* suppressor, standard methods of QTL analysis could not be used (because of the assumption for QTL algorithms that populations have a normal distribution of phenotypes, and there be no selective bias for one phenotype over another).

Out of 463 individuals measured, 96 were homozygous for the *det1-1* (Table 4.2). To accommodate parental controls and a negative control for PCR amplifications in a 96 well PCR plate, only 92 homozygous *det1-1* individuals were genotyped. In lieu of QTL analysis, recombinant break point mapping was used to map the *ted2-1D* suppressor mutation.

Using this data along with the genotypic data, the interval containing *ted2-1D* was initially narrowed to a 508 Kbp region between SSLP markers F19G10 and F21J9 containing 139 predicted protein coding sequences (www.arabidopsis.org). Additional SSLP markers were designed to further narrow the region to a 57 Kbp region containing 13 predicted protein coding sequences in which three can be implicated as likely candidates for *TED2* (Table 4.3).

Table 4.3. Genes in interval between SSLP markers F19G10 and T26J12.1.

Gene Model	Description
AT1G23020.1	ferric-chelate reductase, putative, similar to ferric-chelate reductase (FRO1) (*Pisum sativum*)
*AT1G23030.1	armadillo/beta-catenin repeat family protein / U-box domain-containing protein
AT1G23040.1	hydroxyproline-rich glycoprotein family protein, contains proline-rich domains
AT1G23050.1	hydroxyproline-rich glycoprotein family protein, contains proline-rich extensin domains
AT1G23060.1	expressed protein
AT1G23070.1	hypothetical protein
*AT1G23080.1	auxin efflux carrier protein, putative, similar to efflux carrier of polar auxin transport (*Brassica juncea*)
AT1G23090.1	sulfate transporter, putative, similar to sulfate transporter (*Arabidopsis thaliana*)
AT1G23100.1	10 kDa chaperonin, putative, similar to 10 kDa chaperonin from (*Arabidopsis thaliana*)
AT1G23110.1	hypothetical protein
AT1G23120.1	major latex protein-related / MLP-related, low similarity to major latex protein (*Papaver somniferum*)
AT1G23130.1	Bet v I allergen family protein, similar to Csf-2 (*Cucumis sativus*)
*AT1G23140.1	C2 domain-containing protein, similar to zinc finger and C2 domain protein from (*Arabidopsis thaliana*)

*candidate genes for *TED2*

ted2-1D Suppression of *det1-1* is Overdominant

During the genotyping of individuals in the mapping population for *ted2-1D*, overdominance of the suppressor allele became apparent due to the relationship between genotypes and phenotypes observed. Individuals that were heterozygous for the Col-0 and La-*er* alleles for markers in the interval containing *ted2-1D* had the longest hypocotyls while those homozygous for the Col-0 alleles (homozygous for *ted2-1D*) were medium in length. Since the *ted2-1D* mutation was created in the Col-0 background, those individuals that were homozygous for La-*er* alleles at markers in the interval, thus not containing the *ted2-1D* suppressor mutation, had the shortest hypocotyl length.

There was also a relationship between genotype and phenotype for individuals carrying the *ted2-1D* mutation throughout the vegetative and reproductive phases observed while growing siblings of the 52LH-H-2 genotype used in the cross for the mapping population (Figure 4.2). Individuals were classified as “short”, “medium”, or “tall” based on their rosette size in comparison to the wild type and *det1-1* controls during the vegetative stage (Figure 4.2A) and the extent of apical dominance seen in the reproductive stage (Figure 4.2B). “Small” and “medium” individuals gave rise to “small” and “medium” progeny in the next generation respectively while “tall” individuals gave rise to “tall” and “medium” / “short” individuals (the difference between “medium” and “short” was sometimes difficult to score at earlier stages of development). Based on this data, it was surmised that the 52LH-H-2 line used for developing the mapping populations was homozygous for the *ted2-1D* allele.

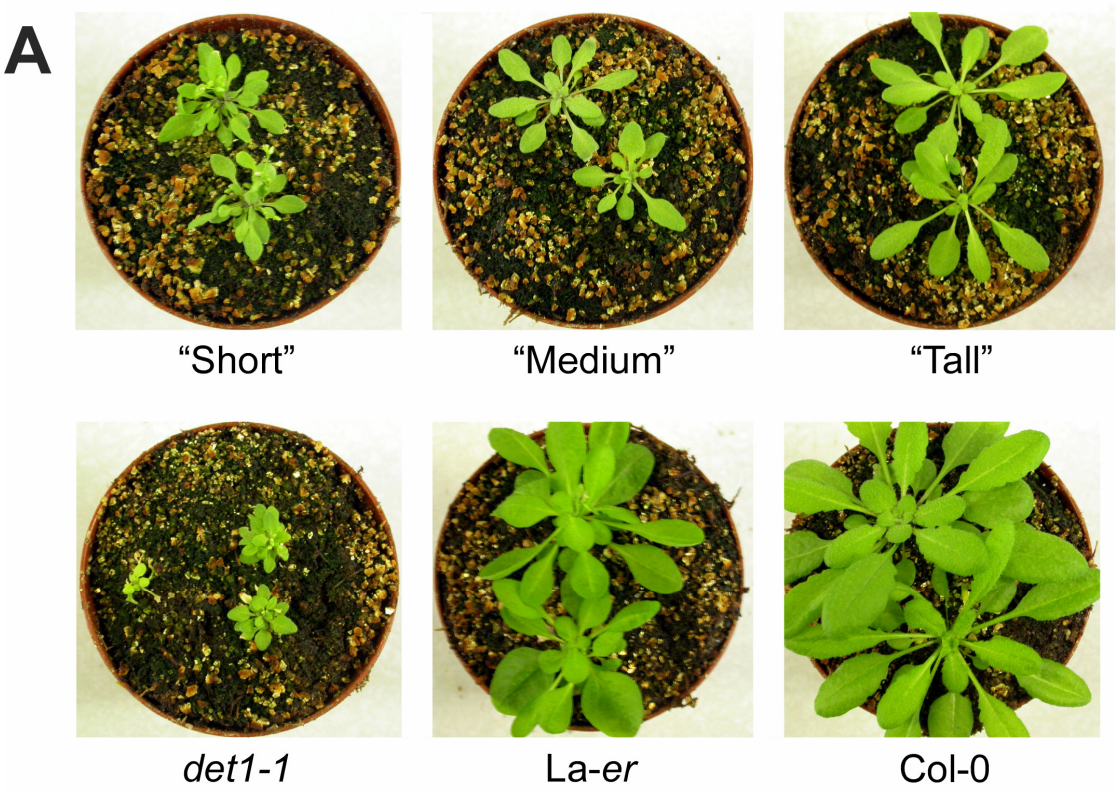


Figure 4.2. *ted2-1D* suppressor overdominance.

(A) "Short", "medium", and "tall" phenotypes of 52LH-H-5 line carrying the *ted2-1D* suppressor at early vegetative stage compared to Col-0, La-*er,* and *det1-1* controls. Photographs are same magnification.

Figure 4.2. Continued.

(B) Phenotypes of adult plants showing restoration of apical dominance by *ted2-1D* suppressor. Plants are siblings from a parent heterozygous for *ted2-1D.* Genotypes shown in photograph are based on relationship seen in mapping *ted2-1D*.

Candidate Genes for *ted2-1D*

Of the 13 genes in the interval between F19G10 and T26J12.1, three genes stood out amongst the others. The armadillo/beta-catenin repeat family protein / U-box domain-containing protein (At1g23023) is a candidate due to the involvement of other U-box domain-containing proteins in post translational regulation of gene expression via the proteosome pathway. The possible interaction of this gene with *DET1* would support the proposed model from the human study where the *DET1* ortholog is involved in targeting proteins for proteosome-dependant degradation. An auxin efflux carrier protein (At1g23080) is also a good candidate for *TED2* due to participation of auxin in cell elongation (which would lead to a longer hypocotyl). Finally, the C2 domain-containing protein (At1g23140) is being considered as a likely candidate since it could be involved in transcriptional regulation of genes involved in photomorphogenesis because of is similarity to C2 domain containing zinc finger transcription factors. Further analysis is needed to determine which gene carries the *ted2-1D* mutation responsible for the suppression of the dark-grown *det1-1* phenotype.

FUTURE PLANS

Future plans to determine which gene carries the *ted2-1D* suppressor consist of two alternative strategies. The first strategy is to sequence the three primary candidate genes from the *ted2-1D* mutant to determine if there are any mutations that would cause a significant change in the protein function. T-DNA constructs containing these candidate genes and their promoter sequences, amplified from the dominant *ted2-1D*

mutant, will be transformed into the *det1-1* mutant to test for suppression of the *det1-1* phenotype. An additionally important experiment is to determine the phenotype of null mutations in each candidate gene using SALK lines containing T-DNA insertions in the candidate genes (Alonso *et al*., 2003). These experiments will confirm which, if any, of these genes carries the *ted2-1D* suppressor mutation, the possible role of *TED2* in photomorphogenesis, and further our understanding of the genetic and regulatory context in which *DET1* is acting.

CHAPTER V

SUMMARY

The primary goal of this research was to further define the role of *DET1* in the regulation of photomorphogenesis by mapping of the *ted1-1SD* and *ted2-1D* extragenic suppressors of the *det1-1* mutation. Using new genomic approaches, most likely candidates for *TED1* and *TED2* were isolated and models were proposed for their participation with *DET1* in the regulation of photomorphogenesis.

The most likely candidate for *TED1* was determined to be the *HAT4* gene based on mapping of a long hypocotyl QTL in an inter-ecotypic cross between the Columbia (Col-0) and Bensheim (Be-0) ecotypes and suppression of the dark-grown *det1-1* phenotype by the Bensheim allele of *HAT4.* Sequence analysis of two distinct Be-0 alleles of *HAT4* (in comparison to Col-0) revealed an unexpected level of functionally- and phenotypically-significant diversity. Expression analysis revealed a previously unknown genetic (and possibly molecular) interaction between *DET1* and *HAT4*.

Models for suppression of the *det1-1* phenotype by the Be-0 alleles of *HAT4* were presented based on sequencing and expression analysis. The first model suggests that the increased level of *HAT4* in the Be-0 genetic background partially suppressed the *det1-1* phenotype in the Col-0 background in an additive manner. A second model suggests the possibility that an increase in the amount of correctly spliced *DET1*

transcript contributed by the Be-0 genetic background in conjunction with the small amount of correctly spliced transcript in the *det1-1* mutant isolated in the Col-0 genetic background partially restores the negative regulation of photomorphogenesis. These findings suggest that there may be high levels of functional genetic variation at the *HAT4* locus within natural populations of Arabidopsis and this diversity may underlie much of the phenotypic diversity in photomorphogenic responses in natural populations.

The most likely candidates for the *ted2-1D* suppressor were narrowed to three genes: 1) an armadillo/beta-catenin repeat family protein / U-box domain-containing similar to other U-box domain-containing proteins involved in post translational regulation of gene expression via the proteosome pathway, 2) an auxin efflux carrier protein due to the role of auxin in cell elongation, and a 3) C2 domain-containing protein possibly involved in transcriptional regulation of genes involved in photomorphogenesis because of is similarity to C2 domain containing zinc finger transcription factors.

Using new genomic methods to isolate and characterize the genes *TED1* and *TED2* has led to a greater understanding of genes involved in the downstream processes of signal transduction and regulation of gene expression during photomorphogenesis. At the same time, these results have also shed light on the interactions of DET1 with other proteins and their roles in the regulation of photomorphogenesis.

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APPENDIX A

SUPPLEMENTARY MATERIAL FOR CHAPTER III

LIGHT SOURCE SPECTRA ANALYSIS

Measuring devices used for light spectra analysis:

LiCor Quantum Photometer (model LI-189)
used to measure μmol m^{-2} s^{-1}

Ocean Optics USB2000 Spectrophotometer
used to measure:
Lumen μJoule cm^{-2}
Lux μWatt cm^{-2}
Candela dBm
μJoule photons
μWattLight emission spectra

Ocean Optics USB2000 Spectrophotometer Specifications:

Detector:	2048-element linear silicon CCD array
Wavelength Range:	200 - 1100 nm
Optical resolution:	~0.3 - 10.0 nm FWHM (depending on grating and size of entrance apeture)
Grating:	#2, UV-VIS, 600 grooves/mm, 650 nm spectral range, 400 nm blaze wavelength, 250-800 nm best efficiency (>30%)
Entrance apeture:	25 μm wide slit
Order-sorting filters:	Variable longpass filter to eliminate second- and third- order effects for systems from 200-850 nm
Focal length:	42 mm (input); 68 mm (output)
Dynamic range:	2 X 10^8 (system); 2000:1 for a single acquisition
Stray light:	<0.05% at 600 nm; <0.10% at 435 nm and 250 nm
Sensitivity:	400 nm -- 90 photons per count; 600 nm -- 41 photons per count; 800 nm -- 203 photons per count
Fiber optic connector:	SMA 905 to 0.22 numerical aperture single-stranded fiber
Data transfer rate:	Full scans into memory every 13 milliseconds with the USB port, every 300 milliseconds with the serial port
Integration time:	3 milliseconds to 65 seconds

Cosine-corrected Irradiance Probe for VIS-NIR region:

Diffusing material:	Opaline glass (350 - 1000 nm)
Disc thickness:	7.9 mm
Barrel dimension:	6.35 mm OD
Sampling geometry:	Accepts light from 180 degree field of view (FOV)
Connector:	SMA 905

Calibration light Source:

LS-1-CAL Radiometric Calibration Standard: VIS-NIR

Medium White Light

Light Source: 2, 34W GE Cool White (FW40CW-RS-WM) bulbs
2, 40W Sylvania Gro Lux (F40/GRO/AQ/WS/RP) bulbs

Filter: 1, 4 mm sheet Lexan

Light intensity: 40 µmol m^{-2} s^{-1}

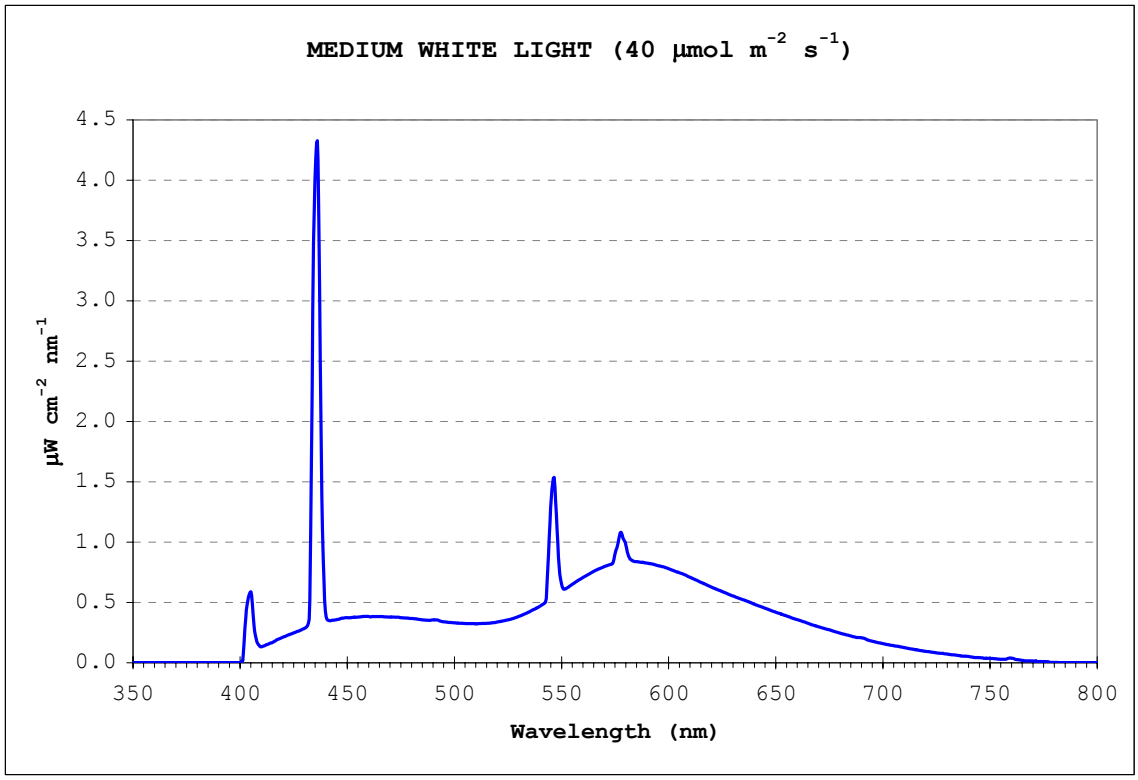

Figure A-1. Spectral output for medium white light source.

Red Light

Light Source: Quantum Devices LED array model SL515-670

Filter: N.A.

Light intensity: 20 µmol m^{-2} s^{-1}

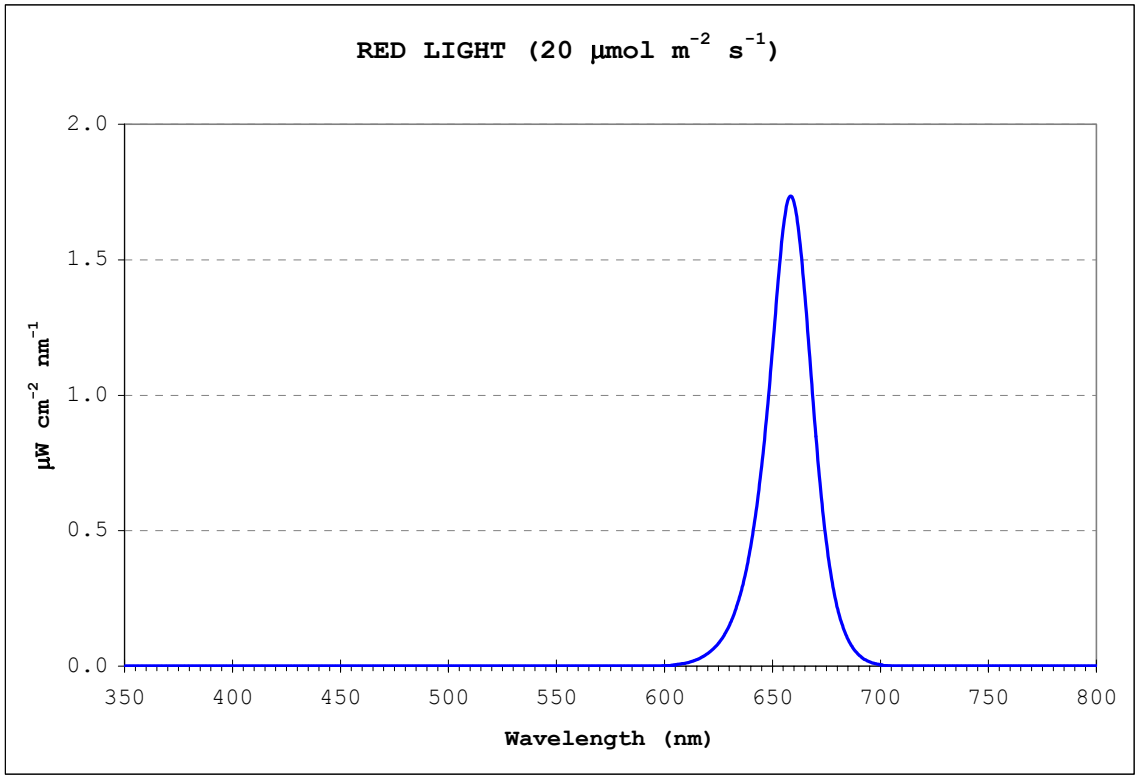

Figure A-2. Spectral output for red light source.

Far-Red Light

Light Source: Quantum Devices LED array model SL515-735

Filter: N.A.

Light intensity: 20 µmol m^{-2} s^{-1}

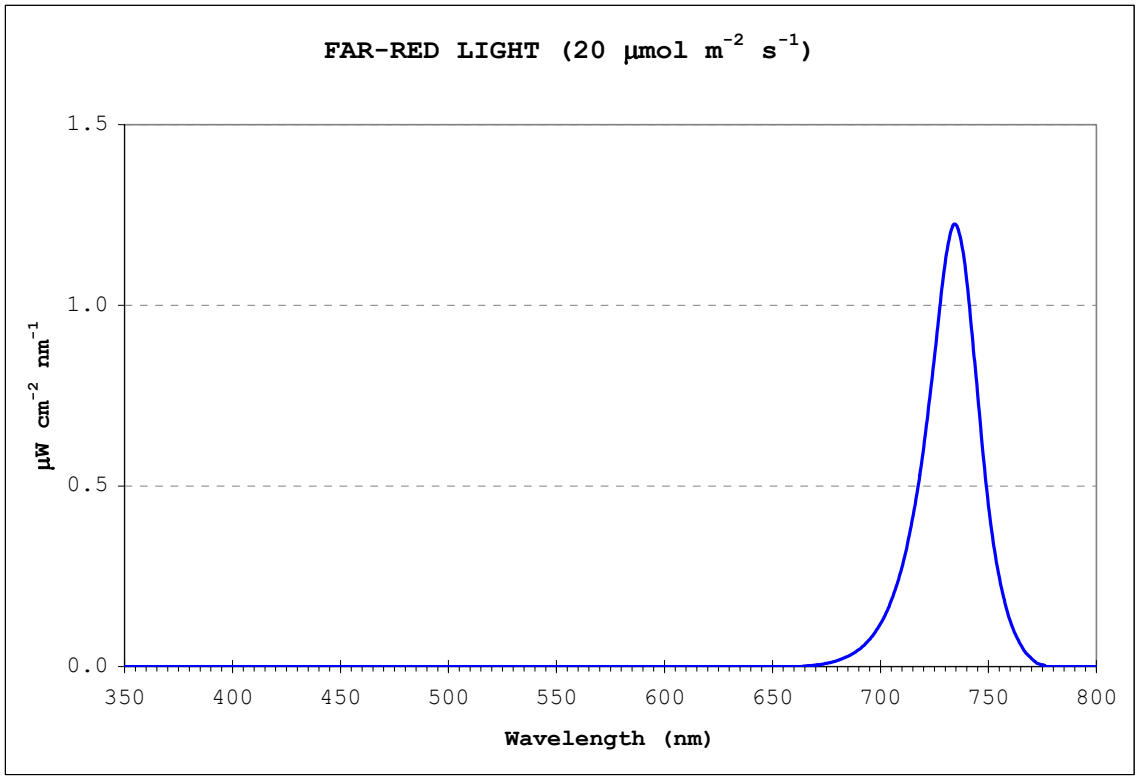

Figure A-3. Spectral output for far-red light source.

Blue Light

Light Source: 4, 20W Coralife® Actinic Blue 7100K (F20 T12 360^0 BP) bulbs

Filters: 1, Lee Neutral Density filter #209
1, Kopp 5-57 blue glass filter

Light intensity: 15 µmol m^{-2} s^{-1}

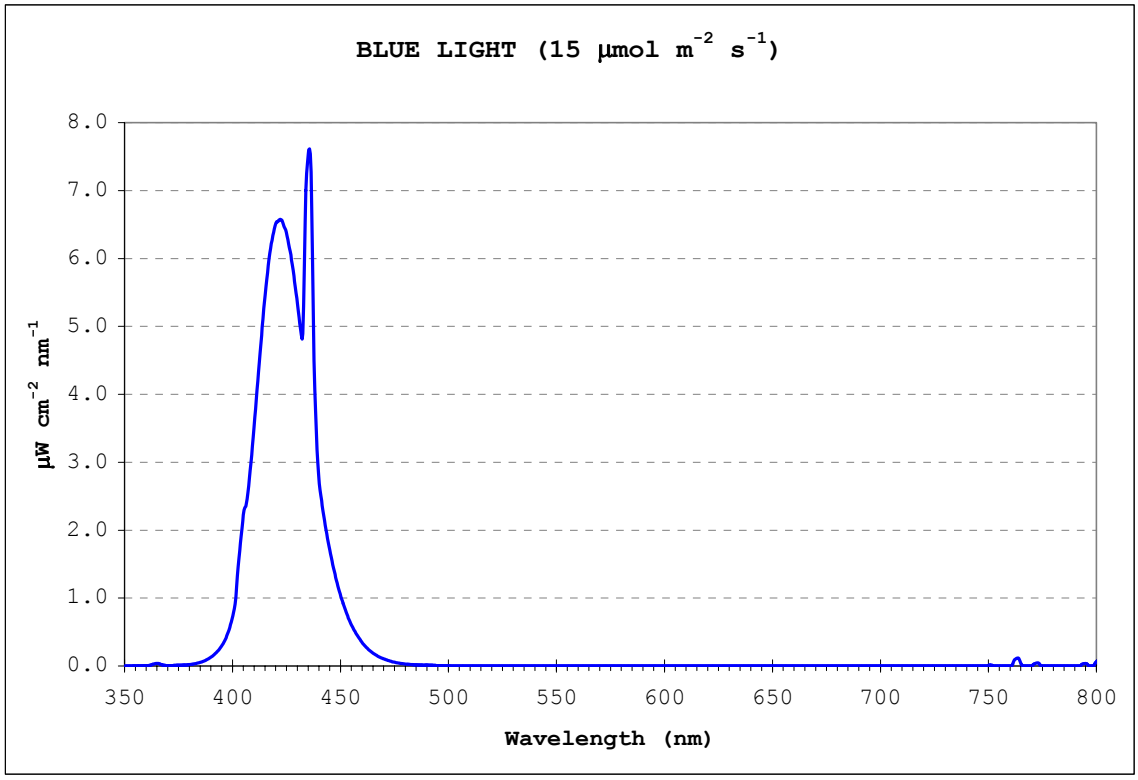

Figure A-4. Spectral output for blue light source.

BENSHEIM (BE-0) ECOTYPE MARKER DATA

Table A-1. SSLP marker information for Be-0 polymorphisms.

Marker	CH	Position	Primers (5' -> 3')	Col-0	Be-0
nga392	I	41.64 cM	F-GGT GTT AAA TGC GGT GTT C R-TTG AAT AAT TTG TAG CCA TG	170 bp	162 bp
nga280	I	83.83 cM	F-GGC TCC ATA AAA AGT GCA CC R-CTG ATC TCA CGG ACA ATA GTG C	86 bp	106 bp
nga111	I	115.55 cM	F-TGT TTT TTA GGA CAA ATG GCG R-CTC CAG TTG GAA GCT AAA GGG	140 bp	128 bp
nga1126	II	50.65 cM	F-GCA CAG TCC AAG TCA CAA CC R-CGC TAC GCT TTT CGG TAA AG	191 bp	193 bp
nga168	II	73.77 cM	F-GAG GAC ATG TAT AGG AGC CTC G R-TCG TCT ACT GCA CTG CCG	144 bp	152 bp
nga162	III	20.56 cM	F-CTC TGT CAC TCT TTT CCT CTG G R-CAT GCA ATT TGC ATC TGA GG	96 bp	106 bp
AtGAPAB	III	43.77 cM	F-CAC CAT GGC TTC GGT TAC TT R-TCC TGA GAA TTC AGT GAA ACC C	142 bp	120 bp
CIW4	III	70.00 cM	F-GTT CAT TAA ACT TGC GTG TGT R-TAC GGT CAG ATT GAG TGA TTC	190 bp	200 bp
FUS6	III	80.00 cM	F-CGG GTA ATT GCG TTT TTG AC R-GGA AGC CTT CAT AAT CGA TAC	75 bp	90 bp
nga6	III	86.41 cM	F-ATG GAG AAG CTT ACA CTG ATC R-TGG ATT TCT TCC TCT CTT CAC	130 bp	144 bp
nga8	IV	26.56 cM	F-TGG CTT TCG TTT ATA AAC ATC C R-GAG GGC AAA TCT TTA TTT CGG	250 bp	154 bp
DET1	IV	31.40 cM	F-CCA ATG ACC CAA TTT TGC R-GGT GAA AAT GGA GGA GAC GA	172 bp	186 bp
132	IV	54.40 cM	F-CAA TAC AAT GTT TGT CAT CCC G R-GAG TAA GTA TTT CAG AGA TAC ACA TC	100 bp	82 bp
FCA6.7	IV	54.45 cM	F-GGT TTC AGT ATC GCA CAA GG R-CTG AAA ATT TGG ATT TAT GTG AAT AAC	86 bp	96 bp
FCA7D2	IV		F-GAC CAC ACA ACT CGA ATA CAA AAT CAA CTT G R-GCT TCA AAT TCA ATA GTT CCT TTT CC	120 bp	148 bp
FCA8.3	IV	57.40 cM	F-ACA CTT CGG CTC CAA GAT AC R-CAG ATT AAA GTG GGT CAG GC	151 bp	121 bp
FCA9.6	IV	58.40 cM	F-CTA TAA GCT TAT CCA TAT GTC TAC C R-GAA TAT ATG CCA GAA AGC CCA G	139 bp	101 bp
T6K21	IV	59.00 cM	F-AAG CGA AAG CGT GTG AGC TAG C R-CTA GAT CGT CCC TCG TCT GAT G	140 bp	80 bp
nga1139	IV	83.41 cM	F-TAG CCG GAT GAG TTG GTA CC R-TTT TTC CTT GTG TTG CAT TCC	114 bp	104 bp
nga1107	IV	104.73 cM	F-GCG AAA AAA CAA AAA AAT CCA R-CGA CGA ATC GAC AGA ATT AGG	150 bp	130 bp
nga225	V	14.31 cM	F-TCT CCC CAC TAG TTT TGT GTC C R-GAA ATC CAA ATC CCA GAG AGG	119 bp	131 bp
nga106	V	33.35 cM	F-GTT ATG GAG TTT CTA GGG CAC G R-TGC CCC ATT TTG TTC TTC TC	157 bp	119 bp
Marker	**CH**	**Position**	**Primers (5' -> 3')**	**Col-0**	**Be-0**

Table A-1. Continued.

Marker	CH	Position	Primers (5' -> 3')	Col-0	Be-0
nga139	V	50.48 cM	F-GGT TTC GTT TCA CTA TCC AGG R-AGA GCT ACC AGA TCC GAT GG	174 bp	184 bp
PHYC	V	71.13 cM	F-CTC AGA GAA TTC CCA GAA AAA TCT R-AAA CTC GAG AGT TTT GTC TAG ATC	207 bp	222 bp
CIW9	V	88.00 cM	F-CAG ACG TAT CAA ATG ACA AAT G R-GAC TAC TGC TCA AAC TAT TCG G	145 bp	133 bp
CIW10	V	115.00 cM	F-CCA CAT TTT CCT TCT TTC ATA R-CAA CAT TTA GCA AAT CAA CTT	140 bp	110 bp

Table A-2. CAPS marker information for Be-0 polymorphisms.

Marker	CH	Position	Primers (5' ->3')	PCR size	Enzyme	Fragments
PHYA	I	11.35 cM	F-CGT CAT GCA AAC TAT CAG TGC TCA AAC CC R-GAT TAC TCA ACC TCA GTG CG	1300 bp	HgaI	Col-0 (1000, 300 bp) Be-0 (1300 bp)
PHYB	II	34.45 cM	F-ATA AAC CAT TAG CCC ACG TG R-CAA TCC TAT GAA GAA TGG CG	1100 bp	Xho I	Col-0 (1100 bp) Be-0 (700, 400 bp)
COP1	II	63.34 cM	F-CCA CTC AGC GCA TCC TTC R-GCT CGG CAT GTG TCA AAA	900 bp	MseI	Col-0 (380, 250, 120, 75 bp) Be-0 (380, 300, 120, 75 bp)
COP9	IV	50.00 cM	F-GAA CAC ATA CTC CGT CAA GC R-CAC AAG TTA GTA GTT GTT GAG ACC	1300 bp	NlaIII	Col-0 (900, 200 bp) Be-0 (900, 400 bp)
SC5	IV	54.20 cM	F-CAC AAG CTA TAC GAT GCT CAC C R-TCG ACG ACT CTC AAG AAC CC	550 bp	AccI	Col-0 (550 bp) Be-0 (300, 250 bp)
HAT4	IV	55.60 bp	F-GTG ACG ATG AAG ATG GTG ATA ACT CC R-GCT CGT AAC CCT AAT TGT TTA GCC	416 bp	EcoRV	Col-0 (360, 56 bp) Be-0 (416 bp)
HY5	V	8.00 cM	F-ATG CAG GAA CAA GCG ACT AGC R-TTT CTC CGC CGG TGT CCT	488 bp	ApoI	Col-0 (300, 134, 54 bp) Be-0 (300, 188 bp)

F_2(TAM793)C MAPPING DATA

Mapping Data for F_2(TAM793)C Population

The following data is in the format used for QTL Cartographer:

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HAT4_CAPS 55.6000
FCA7D2 56.6500
FCA8-3 57.4000
T6K21 59.0000
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-Cross SF2
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a-  10 10
--  -1 .
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-stop individuals markers
-start traits
RawHY_1 10.2000 9.8000 10.4000 8.6000 10.5000 9.4000 9.3000 9.6000
11.0000 10.4000 11.0000 8.6000 12.2000 9.0000 10.6000 11.0000
8.6000 10.0000 11.0000 8.5000 9.6000 11.2000 10.0000 11.0000
8.0000 12.2000 9.4000 10.0000 9.8000 8.5000 12.5000 8.8000
10.0000 9.0000 9.8000 9.6000 10.5000 10.4000 11.4000 8.5000
10.2000 11.6000 11.6000 10.4000 10.2000 10.5000 12.2000 8.8000
9.8000 9.4000 8.0000 9.8000 11.0000 9.6000 9.0000 11.2000
10.6000 9.2000 10.5000 10.0000 9.8000 9.0000 8.0000 8.8000
8.0000 9.1000 9.7000 10.0000 11.1000 10.2000 8.5000 10.2000
7.5000 10.4000 10.0000 10.5000 9.7000 10.2000 9.5000 10.2000
9.6000 10.5000 10.2000 10.0000 11.6000 9.0000 7.8000 7.0000
10.7000 8.6000 11.2000 8.8000 11.8000 11.0000 9.8000 11.5000
9.0000 10.0000 10.4000 9.0000 10.6000 9.1000 9.5000 10.2000
10.8000 9.8000 8.8000 8.7000 11.5000 9.5000 9.5000 11.2000
9.0000 10.0000 9.0000 9.7000 9.0000 10.4000 9.5000 11.5000
9.5000 11.2000 10.0000 10.0000 10.6000 8.6000 9.6000 11.0000
11.0000 12.5000 13.5000 12.5000 11.0000 10.8000 11.5000 10.5000
11.2000 11.0000 9.0000 11.7000 11.0000 12.5000 10.0000 6.2000
10.2000 9.8000 12.0000 9.4000 11.0000 11.5000 10.6000 12.0000
10.8000 8.8000 11.0000 10.0000 12.0000 12.6000 10.8000 10.5000
11.5000 11.0000 9.0000 11.6000 10.6000 12.0000 10.8000 12.2000
11.2000 12.5000 10.4000 10.8000 11.8000 9.2000 11.5000 9.0000
9.8000 11.2000 8.2000 10.5000 9.6000 9.7000 10.5000 9.6000
10.4000 10.2000 10.2000 10.5000 11.0000 9.5000 8.6000 10.0000
11.2000 9.8000 7.0000 8.5000 8.6000 7.6000 9.0000 6.5000
10.0000 10.8000 6.6000 11.0000 10.5000 8.6000 10.0000 8.5000
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9.8000 8.4000 10.5000 11.6000 9.0000 10.5000 10.0000 8.5000
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9.2000 8.4000 9.6000 9.0000 8.0000 8.8000 7.0000 11.6000
9.8000 9.0000 9.7000 9.0000 8.2000 8.0000 10.8000 9.0000
8.0000 10.0000 7.8000 10.0000 8.5000 8.5000 8.0000 10.6000
8.4000 7.2000 9.4000 9.6000 10.0000 9.8000 8.0000 8.2000
9.4000 8.5000 9.8000 8.3000 8.4000 8.0000 6.0000 9.0000
10.5000 10.5000 8.5000 8.4000 8.5000 9.0000 7.6000 9.6000
9.2000 8.0000 8.8000 7.5000 8.6000 9.6000 7.0000 7.8000
9.5000 8.4000 10.6000 9.0000 9.5000 11.0000 9.3000 9.0000
8.8000 8.0000 8.5000 9.4000 9.5000 8.0000 9.0000 11.0000
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9.0000 7.0000 10.5000 11.0000 10.0000 9.8000 9.0000 9.8000
11.0000 7.8000 9.0000 10.5000 9.8000 10.8000 8.8000 10.5000
8.5000 7.5000 9.0000 10.5000 10.6000 10.5000 9.5000 10.0000
8.2000 9.4000 7.0000 9.8000 8.8000 9.0000 6.8000 10.0000
8.0000 9.5000 9.0000 9.5000 8.0000 10.5000 8.5000 8.2000
9.5000 7.8000 9.0000 7.4000 9.4000 9.0000 10.4000 7.3000
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9.5000 9.4000 8.5000 9.0000 7.0000 10.4000 10.5000 8.8000
8.6000 8.0000 8.2000 9.0000 9.0000 11.2000 11.0000 9.4000
9.8000 9.0000 8.6000 9.8000 9.5000 10.0000 8.6000 9.4000
9.5000 8.8000 8.2000 11.0000 11.5000 11.5000 8.6000 9.0000
9.2000 10.8000 7.5000 7.6000 8.5000 8.6000 9.4000 8.5000
11.2000 9.5000 9.8000 10.0000
NormHY_2 1.2905 1.2399 1.3158 1.0880 1.3284 1.1892 1.1766 1.2145
1.3917 1.3158 1.3917 1.0880 1.5435 1.1386 1.3411 1.3917
1.0880 1.2652 1.3917 1.0754 1.2145 1.4170 1.2652 1.3917
1.0121 1.5435 1.1892 1.2652 1.2399 1.0754 1.5814 1.1133
1.2652 1.1386 1.2399 1.2145 1.3284 1.3158 1.4423 1.0754
1.2905 1.4676 1.4676 1.3158 1.2905 1.3284 1.5435 1.1133
1.2399 1.1892 1.0121 1.2399 1.3917 1.2145 1.1386 1.4170
1.3411 1.1639 1.3284 1.2652 1.2399 1.1386 1.0121 1.1133
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1.0959 1.2177 1.2664 1.0959 1.2907 1.1081 1.1568 1.2420
1.3151 1.1933 1.0715 1.0594 1.4003 1.1568 1.1568 1.3638
1.0959 1.2177 1.0959 1.1811 1.0959 1.2664 1.1568 1.4003
1.1568 1.3638 1.2177 1.2177 1.2907 1.0472 1.1689 1.3394
1.3394 1.5221 1.6438 1.5221 1.3394 1.3151 1.4003 1.2785
1.3638 1.3394 1.0959 1.4247 1.3394 1.5221 1.2177 0.7549
1.2420 1.1933 1.4612 1.1446 1.3394 1.4003 1.2907 1.4612
1.3151 1.0715 1.3394 1.2177 1.4612 1.5342 1.3151 1.2785
1.4003 1.3394 1.0959 1.4125 1.2907 1.4612 1.3151 1.4855
1.3638 1.5221 1.2664 1.3151 1.4368 1.1202 1.4003 1.0959
1.1933 1.3638 1.1445 1.4655 1.3399 1.3539 1.4655 1.3399
1.4516 1.4236 1.4236 1.4655 1.5353 1.3259 1.2003 1.3957
1.5632 1.3678 0.9770 1.1864 1.2003 1.0608 1.2562 0.9072
1.3957 1.5074 0.9212 1.5353 1.4655 1.2003 1.3957 1.1864
1.3259 1.3957 1.3957 1.3678 1.2841 1.3957 1.3120 1.1724
1.3678 1.1724 1.4655 1.6190 1.2562 1.4655 1.3957 1.1864
1.0468 1.4655 1.2562 1.2562 1.4655 1.1864 1.3399 1.1724
1.2841 1.1724 1.3399 1.2562 1.1166 1.2282 0.9770 1.6190
1.3678 1.2562 1.3539 1.2562 1.1445 1.1166 1.5074 1.2562
1.1166 1.3957 1.0887 1.3957 1.1864 1.1864 1.1166 1.4795
1.1724 1.0049 1.3120 1.3399 1.3957 1.3678 1.1166 1.1445
1.3120 1.1864 1.3235 1.1209 1.1344 1.0804 0.8103 1.2154
1.4180 1.4180 1.1479 1.1344 1.1479 1.2154 1.0264 1.2965
1.2424 1.0804 1.1884 1.0129 1.1614 1.2965 0.9453 1.0534
1.2830 1.1344 1.4315 1.2154 1.2830 1.4855 1.2559 1.2154
1.1884 1.0804 1.1479 1.2695 1.2830 1.0804 1.2154 1.4855
1.2154 0.9453 1.4180 1.4855 1.3505 1.3235 1.2154 1.3235
1.4855 1.0534 1.2154 1.4180 1.3235 1.4585 1.1884 1.4180
1.1479 1.0129 1.2154 1.4180 1.4315 1.4180 1.2830 1.3505

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1.1074 1.2695 0.9453 1.3235 1.1884 1.2154 0.9183 1.3505
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1.2830 1.0534 1.2154 0.9994 1.2695 1.2154 1.4045 0.9859
1.3097 1.3097 1.2069 1.2840 1.5408 1.4124 1.3097 1.1042
1.2198 1.2069 1.0914 1.1556 0.8988 1.3353 1.3482 1.1299
1.1042 1.0272 1.0529 1.1556 1.1556 1.4381 1.4124 1.2069
1.2583 1.1556 1.1042 1.2583 1.2198 1.2840 1.1042 1.2069
1.2198 1.1299 1.0529 1.4124 1.4766 1.4766 1.1042 1.1556
1.1813 1.3867 0.9630 0.9758 1.0914 1.1042 1.2069 1.0914
1.4381 1.2198 1.2583 1.2840
-stop traits
#endcross
-quit
-end
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Mapping Data for F_2(TAM793)C – Repeat Population

The following data is in the format used for QTL Cartographer:

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#FileID F2(TAM793)C TED1 ALL
#bychromosome
-type position
-function 2
-Units cM
-chromosomes 1
-maximum 4
-named yes
-start
-Chromosome CHR_IV 4
FCA6-7 54.4500
HAT4_CAPS 55.6000
FCA7D2 56.6500
FCA8-3 57.4000
-stop
#endchromosome
---------------------------------------
#bycross
-Samplesize 304
-Cross SF2
-traits 2
-missingtrait .
-case yes
-TranslationTable
AA  2  2
Aa  1  1
aa  0  0
A-  12 12
a-  10 10
--  -1 .
-start individuals markers
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ID-501 1 . . 1
ID-502 1 . . 1
ID-503 2 . . 2
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ID-507 1 . . 1
ID-508 2 . . 2
ID-509 0 . . 0
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```

```
ID-788 2 . . 2
ID-789 2 . . 2
ID-790 2 . . 2
ID-791 1 . . 1
ID-792 1 . . 1
ID-793 2 . . 2
ID-794 1 . . 1
ID-795 1 . . 1
ID-796 1 . . 1
ID-797 1 . . 1
ID-798 2 . . 2
ID-799 0 . . 0
ID-800 0 . . 0
ID-801 2 . . 2
ID-802 1 . . 1
ID-803 1 . . 1
-stop individuals markers
-start traits
RawHY_1 6.4000 5.0000 4.5000 3.5000 3.0000 3.6000 3.2000 3.0000
7.0000 4.4000 7.6000 7.2000 4.5000 4.4000 5.8000 6.2000
4.4000 4.4000 4.8000 5.8000 5.8000 4.8000 4.8000 3.4000
4.6000 5.5000 4.2000 5.5000 5.0000 5.0000 4.4000 5.4000
3.6000 3.8000 3.2000 3.0000 3.4000 7.5000 6.6000 5.6000
6.2000 3.5000 4.5000 3.6000 4.0000 6.0000 4.6000 3.9000
7.2000 6.6000 4.0000 6.5000 7.3000 5.7000 4.6000 7.0000
5.8000 4.5000 5.0000 5.6000 6.2000 3.8000 6.6000 6.0000
3.8000 3.1000 6.0000 3.5000 6.0000 4.4000 4.0000 5.8000
3.5000 5.2000 4.0000 4.6000 4.8000 4.0000 6.5000 4.4000
3.5000 3.0000 7.4000 4.8000 4.4000 4.2000 3.2000 4.4000
5.6000 3.5000 6.0000 4.0000 8.0000 4.6000 4.5000 4.6000
3.8000 3.5000 4.5000 4.5000 3.8000 6.0000 3.2000 6.2000
4.0000 5.0000 3.5000 4.5000 5.6000 5.8000 7.0000 5.0000
4.5000 4.5000 7.5000 3.6000 4.4000 6.8000 6.4000 3.4000
4.0000 2.5000 8.6000 3.8000 4.8000 5.2000 2.8000 6.5000
6.5000 5.2000 7.1000 3.5000 6.2000 2.6000 5.0000 3.8000
5.8000 5.0000 3.0000 5.0000 3.4000 4.2000 8.0000 3.6000
8.0000 7.0000 4.0000 4.8000 2.6000 4.5000 4.0000 3.5000
3.0000 5.5000 4.4000 6.2000 6.8000 6.5000 4.2000 5.6000
3.5000 6.6000 4.6000 3.4000 6.0000 5.5000 5.4000 6.5000
7.2000 4.4000 4.4000 7.0000 7.5000 5.8000 7.5000 5.5000
4.0000 7.5000 9.0000 8.8000 2.6000 3.4000 2.8000 4.4000
5.2000 6.6000 6.6000 5.0000 9.4000 4.5000 4.2000 4.4000
5.6000 4.2000 7.2000 6.2000 6.6000 6.8000 6.2000 3.0000
4.6000 3.0000 6.6000 2.8000 4.0000 6.2000 8.4000 7.2000
6.6000 5.0000 8.6000 6.0000 7.6000 9.5000 4.6000 7.2000
8.8000 7.8000 7.2000 7.8000 8.4000 3.6000 4.5000 6.8000
6.4000 9.8000 7.5000 6.8000 5.6000 4.0000 4.5000 5.0000
7.0000 9.4000 6.0000 4.4000 4.0000 6.0000 5.2000 8.4000
4.6000 7.4000 9.2000 8.4000 6.8000 7.0000 2.8000 6.5000
6.2000 7.5000 5.0000 5.4000 6.0000 6.6000 7.0000 5.8000
8.2000 4.0000 8.6000 4.9000 9.1000 4.0000 5.8000 6.5000
8.5000 3.5000 4.5000 5.5000 11.5000 9.2000 4.5000 6.5000
6.5000 5.8000 6.0000 9.8000 5.0000 5.5000 8.8000 6.5000
7.0000 7.5000 8.0000 3.0000 3.2000 3.0000 6.6000 4.5000
```

```
5.4000 4.6000 8.0000 5.8000 7.0000 7.0000 6.5000 6.0000
3.8000 4.8000 7.8000 4.6000 3.0000 6.0000 5.0000 4.6000

NormHY_2 2.2519 1.7593 1.5833 1.2315 1.0556 1.2667 1.1259 1.0556
2.4630 1.5481 2.6741 2.5333 1.5833 1.5481 2.0407 2.1815
1.5481 1.5481 1.6889 2.0407 2.0407 1.6889 1.6889 1.1963
1.6185 1.9352 1.4778 1.9352 1.7593 1.7593 1.5481 1.9000
1.2667 1.3370 1.1259 1.0556 1.1963 2.6389 2.3222 1.9704
2.1815 1.2315 1.5833 1.2667 1.4074 2.1111 1.6185 1.3722
2.5333 2.3222 1.4074 2.2870 2.5685 2.0056 1.6185 2.4630
2.0407 1.5833 1.7593 1.9704 2.1815 1.3370 2.3222 2.1111
1.3370 1.0907 2.1111 1.2315 2.1111 1.5481 1.4074 2.0407
1.2315 1.8296 1.4074 1.6185 1.6889 1.4074 2.2870 1.5481
1.2315 1.0556 2.6037 1.6889 1.5481 1.4778 1.1259 1.5481
1.9704 1.2315 2.1111 1.4074 2.8148 1.6185 1.5833 1.6185
1.3370 1.2315 1.5833 1.5833 1.3370 1.9068 1.0169 1.9703
1.2712 1.5890 1.1123 1.4301 1.7797 1.8432 2.2246 1.5890
1.4301 1.4301 2.3835 1.1441 1.3983 2.1610 2.0339 1.0805
1.2712 0.7945 2.7331 1.2076 1.5254 1.6525 0.8898 2.0657
2.0657 1.6525 2.2564 1.1123 1.9703 0.8263 1.5890 1.2076
1.8432 1.5890 0.9534 1.5890 1.0805 1.3347 2.5424 1.1441
2.5424 2.2246 1.2712 1.5254 0.8263 1.4301 1.2712 1.1123
0.9534 1.7479 1.3983 1.9703 2.1610 2.0657 1.3347 1.7797
1.1123 2.0975 1.4619 1.0805 1.9068 1.7479 1.7161 2.0657
2.2881 1.3983 1.3983 2.2246 2.3835 1.8432 2.3835 1.7479
1.2712 2.3835 2.8602 2.7966 0.8263 1.0805 0.8898 1.3983
1.6525 2.0975 2.0975 1.5890 2.9873 1.4301 1.3347 1.3983
1.7797 1.3347 2.2881 1.9703 2.0975 2.1610 1.9703 0.9534
1.4619 0.9534 2.0975 0.8898 1.2712 1.5860 2.1488 1.8419
1.6884 1.2791 2.2000 1.5349 1.9442 2.4302 1.1767 1.8419
2.2512 1.9953 1.8419 1.9953 2.1488 0.9209 1.1512 1.7395
1.6372 2.5070 1.9186 1.7395 1.4326 1.0233 1.1512 1.2791
1.7907 2.4047 1.5349 1.1256 1.0233 1.5349 1.3302 2.1488
1.1767 1.8930 2.3535 2.1488 1.7395 1.7907 0.7163 1.6628
1.5860 1.9186 1.2791 1.3814 1.5349 1.6884 1.7907 1.4837
2.0977 1.0233 2.2000 1.2535 2.3279 1.0233 1.4837 1.6628
2.1744 0.8953 1.1512 1.4070 2.9419 2.3535 1.1512 1.6628
1.6628 1.4837 1.5349 2.5070 1.2791 1.4070 2.2512 1.6628
1.7907 1.9186 2.0465 0.7674 0.8186 0.7674 1.6884 1.1512
1.3814 1.1767 2.0465 1.4837 1.7907 1.7907 1.6628 1.5349
0.9721 1.2279 1.9953 1.1767 0.7674 1.5349 1.2791 1.1767

-stop traits
#endcross
-quit
-end
```

F_2(TAM826)A MAPPING DATA

Mapping Data for F_2(TAM826)A Mapping Population

The following data is in the format used for QTL Cartographer:

```
#FileID F2(TAM826)A. F2 individuals grown in dark and homozygous for
det1-1 mutation.
#bychromosome
-type position
-function 2
-Units cM
-chromosomes 5
-maximum 10
-named yes
-start
-Chromosome CHR_I 4
PHYA 10.0000
nga392 39.5200
nga280 81.7000
nga111 113.0000
-Chromosome CHR_II 4
PHYB 34.5000
nga1126 50.7000
COP1 63.3000
nga168 73.7700
-Chromosome CHR_III 5
nga162 20.5000
AtGAPAB 43.7700
CIW4 70.0000
FUS6 80.0000
nga6 86.4000
-Chromosome CHR_IV 10
nga8 26.6000
DET1 31.4000
COP9 50.0000
SC5 54.2000
FCA6_7 54.4500
HAT4_CAPS 55.6000
FCA7D2 56.6500
T6K21 59.0000
nga1139 83.4000
nga1107 105.0000
-Chromosome CHR_V 7
HY5 8.0000
nga225 26.6000
nga106 31.4000
nga139 50.4800
PHYC 71.1000
CIW9 88.0000
CIW10 115.0000
-stop
#endchromosome
```

```
---------------------------------------
#bycross
-Samplesize 44
-Cross SF2
-traits 1
-missingtrait .
-case yes
-TranslationTable
AA  2  2
Aa  1  1
aa  0  0
A-  12 12
a-  10 10
--  -1 .
-start individuals markers
IND-056 . . 0 . . 1 1 2 1 1 0 . 1 0 0 0 . 0 0 . 0 1 1 1 1 1 2 . 2 1
IND-133 . . 2 . . 1 1 1 0 0 0 . 0 0 0 1 1 1 1 2 1 1 1 0 0 0 0 . 1 1
IND-088 . . 1 . . 0 0 0 1 1 0 . 0 . 0 0 0 0 0 0 0 1 1 2 2 2 2 . 1 1
IND-097 . . 0 . . 1 1 1 2 2 1 . 1 0 0 0 0 0 0 0 0 0 0 2 2 1 0 . 0 0
IND-034 . . 2 . . 1 2 2 1 2 2 . 2 0 0 0 1 1 1 1 1 1 1 0 0 0 0 . 0 0
IND-036 . . 2 . . 1 1 1 2 1 1 . 1 0 0 0 0 0 0 0 0 1 0 0 1 1 1 0 . 0 0
IND-043 . . 1 . . 0 1 1 0 1 1 . 0 0 0 0 0 0 0 0 0 0 1 1 0 0 0 0 . 1 1
IND-077 . . 2 . . 2 . 2 1 1 . . 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 1 . 1 1
IND-086 . . 2 . . 1 1 1 2 1 0 . 0 0 0 0 0 0 0 0 0 0 . 2 1 1 1 1 . 1 2
IND-090 . . 2 . . 1 . 2 2 2 1 . 1 0 0 . 0 0 0 0 0 0 0 1 1 1 1 . 0 0
IND-115 . . 0 . . 1 1 1 2 2 2 . 2 0 0 1 1 1 1 1 2 2 1 1 2 1 0 . 2 2
IND-044 . . 0 . . 1 2 2 1 1 0 . 1 0 0 0 0 0 0 0 0 0 2 2 0 1 2 1 . 1 0
IND-013 . . 0 . . 0 0 0 1 1 1 . 1 0 0 0 0 0 0 0 0 . 1 1 . 1 2 0 . 2 1
IND-152 . . 2 . . 2 2 2 1 1 2 . 2 0 0 0 0 0 0 0 0 0 1 1 . 2 2 2 . 2 2
IND-157 . . 0 . . 1 1 1 2 1 1 . 1 0 0 1 1 1 1 1 1 1 1 1 . 2 2 2 . 1 0
IND-161 . . 1 . . 1 1 1 1 2 2 . 1 0 0 0 0 0 0 0 1 0 1 1 1 1 1 1 . 0 0
IND-162 . . 0 . . 1 1 1 2 1 1 . 1 0 0 . 0 0 0 0 0 0 0 0 0 0 0 0 . 1 1
IND-153 . . 2 . . 2 2 2 2 1 1 . 1 0 0 1 1 1 1 1 1 1 2 0 1 1 1 . 1 2
IND-028 . . 2 . . 1 2 2 1 1 2 . 1 0 0 0 0 0 0 0 0 1 . 1 1 2 2 1 . 1 1
IND-046 . . 0 . . 2 2 2 2 2 1 . 1 0 0 0 0 0 0 0 0 0 0 0 1 1 1 1 . 1 1
IND-095 . . 1 . . 2 2 1 0 0 1 . 2 0 0 0 0 0 0 0 0 0 0 0 0 0 . 0 . 1 1
IND-017 . . 0 . . 1 1 1 1 1 0 . 0 0 0 0 0 0 1 0 1 0 0 0 1 1 0 0 . 2 1
IND-022 . . 2 . . 1 1 2 2 2 2 . 2 0 0 0 0 0 0 0 0 0 0 0 2 2 2 2 . 2 2
IND-092 . . 0 . . 2 0 1 1 1 1 . 1 0 0 . 0 . . 0 0 0 0 0 0 0 0 . 0 0
IND-010 . . 1 . . 1 1 1 0 0 0 . 0 1 0 0 0 0 0 0 0 0 1 2 2 2 2 . 2 1
IND-033 . . 1 . . 1 1 1 1 1 0 . 0 0 0 1 1 1 1 1 1 2 2 1 1 2 2 . 1 1
IND-155 . . 0 . . 1 . 1 1 1 0 . 0 0 0 0 0 0 0 0 0 0 0 1 1 0 0 . 1 1
IND-003 . . 1 . . 1 1 1 0 0 1 . 1 0 0 0 0 0 0 0 0 1 1 2 0 . 1 1 . 1 1
IND-012 . . 1 . . 0 2 2 0 0 1 . 1 0 0 0 0 0 1 1 1 2 1 0 1 1 1 1 . 2 2
IND-091 . . 2 . . 1 1 1 . 0 2 . 2 0 0 1 1 0 1 0 1 2 2 0 0 0 0 . 2 2
IND-100 . . 1 . . 1 1 1 0 1 1 . 1 0 0 . 0 0 0 0 0 0 0 1 1 0 0 . . 1
IND-148 . . 1 . . 2 1 1 1 1 2 . 1 0 0 0 1 2 1 2 1 1 1 0 0 0 0 . 1 2
IND-009 . . 1 . . 1 . 1 1 1 1 . 1 0 0 . 1 1 1 0 1 1 1 1 0 1 1 . 2 2
IND-011 . . 2 . . 2 0 0 1 1 2 . 1 0 0 1 1 1 1 1 2 1 1 1 1 . 1 . . 2
IND-016 . . 1 . . 1 1 1 0 0 0 . 0 0 0 1 1 1 1 1 1 1 1 . 0 0 0 . 1 1
IND-089 . . 1 . . 1 1 1 0 0 0 . 0 0 0 1 1 1 1 1 1 1 1 1 1 1 1 . 1 0
IND-005 . . 1 . . 2 . 2 1 1 1 . 1 0 0 0 0 0 0 0 0 0 0 2 2 2 2 . 2 0
IND-039 . . 0 . . 1 1 1 0 0 1 . 1 0 0 0 1 1 1 1 0 1 1 1 0 1 1 . 2 2
IND-001 . . 0 . . 2 2 2 1 1 1 . 1 0 0 . 1 1 1 1 1 1 1 1 1 1 1 . 1 0
```

```
IND-002 . . 1 . . 1 . 1 1 0 . . 1 0 0 1 2 2 2 . 2 1 1 1 1 2 2 . . .
IND-004 . . 1 . . 1 1 1 1 1 1 . 1 0 0 . 1 1 1 1 1 1 1 0 0 0 0 . 0 1
IND-083 . . 1 . . 1 . 1 0 1 . . 0 0 0 . . 1 1 1 1 1 1 1 1 1 1 . 1 2
IND-093 . . 1 . . 0 1 1 0 0 0 . 1 0 0 1 1 1 1 0 1 2 2 0 0 0 0 . 1 1
IND-051 . . 1 . . 1 1 1 0 1 1 . 1 0 0 1 1 1 2 1 2 2 2 1 1 1 1 . 2 1
-stop individuals markers
-start traits
RawHY_1 1.5000 1.5000 1.8000 1.8000 2.0000 2.0000 2.0000 2.0000
2.0000 2.0000 2.0000 2.2000 2.3000 2.5000 2.5000 2.5000
2.5000 2.8000 3.0000 3.0000 3.0000 4.1000 4.2000 4.2000
4.5000 4.5000 4.5000 4.7000 5.0000 5.0000 5.0000 5.0000
5.1000 5.2000 5.2000 5.2000 5.5000 5.5000 5.6000 5.8000
6.0000 6.0000 6.0000 7.0000
-stop traits
#endcross
-quit
-end
```

Mapping Data for F_2(TAM826)A – Repeat Mapping Population

The following data is in the format used for QTL Cartographer:

```
#FileID  F2(TAM826)A TED1
#bychromosome
-type  position
-function  2
-Units  cM
-chromosomes  5
-maximum 10
-named yes
-start
-Chromosome  CHR_I  4
PHYA  10.0000
nga392  39.5200
nga280  81.7000
nga111  113.0000
-Chromosome  CHR_II  4
PHYB  34.5000
nga1126  50.7000
COP1  63.3000
nga168  73.7700
-Chromosome  CHR_III  5
nga162  20.5000
AtGAPAB  43.7700
CIW4  70.0000
FUS6  80.0000
nga6  86.4000
-Chromosome  CHR_IV  10
nga8  26.6000
DET1  31.4000
COP9  50.0000
SC5  54.2000
FCA6_7  54.4500
HAT4_CAPS  55.6000
FCA7D2  56.6500
T6K21  59.0000
nga1139  83.4000
nga1107  105.0000
-Chromosome  CHR_V  7
HY5  8.0000
nga225  26.6000
nga106  31.4000
nga139  50.4800
PHYC  71.1000
CIW9  88.0000
CIW10  115.0000
-stop
#endchromosome
----------------------------------------
#bycross
-Samplesize  44
```

```
-Cross  SF2
-traits  1
-missingtrait  .
-case yes
-TranslationTable
AA  2  2
Aa  1  1
aa  0  0
A-  12 12
a-  10 10
--  -1 .
-start individuals markers
IND-056 . . 0 . . 1 1 2 1 1 0 . 1 0 0 0 . 0 0 . 0 1 1 1 1 1 2 . 2 1
IND-133 . . 2 . . 1 1 1 0 0 0 . 0 0 0 1 1 1 1 2 1 1 1 0 0 0 0 . 1 1
IND-088 . . 1 . . 0 0 0 1 1 0 . 0 . 0 0 0 0 0 0 0 1 1 2 2 2 2 . 1 1
IND-097 . . 0 . . 1 1 1 2 2 1 . 1 0 0 0 0 0 0 0 0 0 0 2 2 1 0 . 0 0
IND-034 . . 2 . . 1 2 2 1 2 2 . 2 0 0 0 1 1 1 1 1 1 1 0 0 0 0 . 0 0
IND-036 . . 2 . . 1 1 1 2 1 1 . 1 0 0 0 0 0 0 0 1 0 0 1 1 1 0 . 0 0
IND-043 . . 1 . . 0 1 1 0 1 1 . 0 0 0 0 0 0 0 0 0 1 1 0 0 0 0 . 1 1
IND-077 . . 2 . . 2 . 2 1 1 . . 0 0 0 0 0 0 0 0 0 0 0 0 0 0 1 . 1 1
IND-086 . . 2 . . 1 1 1 2 1 0 . 0 0 0 0 0 0 0 0 0 . 2 1 1 1 1 . 1 2
IND-090 . . 2 . . 1 . 2 2 2 1 . 1 0 0 . 0 0 0 0 0 0 0 1 1 1 1 . 0 0
IND-115 . . 0 . . 1 1 1 2 2 2 . 2 0 0 1 1 1 1 1 2 2 1 1 2 1 0 . 2 2
IND-044 . . 0 . . 1 2 2 1 1 0 . 1 0 0 0 0 0 0 0 0 2 2 0 1 2 1 . 1 0
IND-013 . . 0 . . 0 0 0 1 1 1 . 1 0 0 0 0 0 0 0 0 . 1 1 . 1 2 0 . 2 1
IND-152 . . 2 . . 2 2 2 1 1 2 . 2 0 0 0 0 0 0 0 0 0 1 1 . 2 2 2 . 2 2
IND-157 . . 0 . . 1 1 1 2 1 1 . 1 0 0 1 1 1 1 1 1 1 1 1 . 2 2 2 . 1 0
IND-161 . . 1 . . 1 1 1 1 2 2 . 1 0 0 0 0 0 0 0 1 0 1 1 1 1 1 1 . 0 0
IND-162 . . 0 . . 1 1 1 2 1 1 . 1 0 0 . 0 0 0 0 0 0 0 0 0 0 0 0 . 1 1
IND-153 . . 2 . . 2 2 2 2 1 1 . 1 0 0 1 1 1 1 1 1 1 2 0 1 1 1 . 1 2
IND-028 . . 2 . . 1 2 2 1 1 2 . 1 0 0 0 0 0 0 0 0 1 . 1 1 2 2 1 . 1 1
IND-046 . . 0 . . 2 2 2 2 2 1 . 1 0 0 0 0 0 0 0 0 0 0 0 1 1 1 1 . 1 1
IND-095 . . 1 . . 2 2 1 0 0 1 . 2 0 0 0 0 0 0 0 0 0 0 0 0 0 . 0 . 1 1
IND-017 . . 0 . . 1 1 1 1 1 0 . 0 0 0 0 0 0 1 0 1 0 0 0 1 1 0 0 . 2 1
IND-022 . . 2 . . 1 1 2 2 2 2 . 2 0 0 0 0 0 0 0 0 0 0 0 2 2 2 2 . 2 2
IND-092 . . 0 . . 2 0 1 1 1 1 . 1 0 0 . 0 . . 0 0 0 0 0 0 0 0 . 0 0
IND-010 . . 1 . . 1 1 1 0 0 0 . 0 1 0 0 0 0 0 0 0 0 0 1 2 2 2 2 . 2 1
IND-033 . . 1 . . 1 1 1 1 1 0 . 0 0 0 1 1 1 1 1 1 1 2 2 1 1 2 2 . 1 1
IND-155 . . 0 . . 1 . 1 1 1 0 . 0 0 0 0 0 0 0 0 0 0 0 0 1 1 0 0 . 1 1
IND-003 . . 1 . . 1 1 1 0 0 1 . 1 0 0 0 0 0 0 0 0 1 1 2 0 . 1 1 . 1 1
IND-012 . . 1 . . 0 2 2 0 0 1 . 1 0 0 0 0 0 1 1 1 2 1 0 1 1 1 1 . 2 2
IND-091 . . 2 . . 1 1 1 . 0 2 . 2 0 0 1 1 0 1 0 1 2 2 2 0 0 0 0 . 2 2
IND-100 . . 1 . . 1 1 1 0 1 1 . 1 0 0 . 0 0 0 0 0 0 0 0 1 1 0 0 . . 1
IND-148 . . 1 . . 2 1 1 1 1 2 . 1 0 0 0 0 1 2 1 2 1 1 1 0 0 0 0 . 1 2
IND-009 . . 1 . . 1 . 1 1 1 1 . 1 0 0 . 1 1 1 0 1 1 1 1 0 1 1 . 2 2
IND-011 . . 2 . . 2 0 0 1 1 2 . 1 0 0 1 1 1 1 1 2 1 1 1 1 . 1 . . 2
IND-016 . . 1 . . 1 1 1 0 0 0 . 0 0 0 1 1 1 1 1 1 1 1 . 0 0 0 . 1 1
IND-089 . . 1 . . 1 1 1 0 0 0 . 0 0 0 1 1 1 1 1 1 1 1 1 1 1 1 . 1 0
IND-005 . . 1 . . 2 . 2 1 1 1 . 1 0 0 0 0 0 0 0 0 0 0 2 2 2 2 . 2 0
IND-039 . . 0 . . 1 1 1 0 0 1 . 1 0 0 0 1 1 1 1 0 1 1 1 0 1 1 . 2 2
IND-001 . . 0 . . 2 2 2 1 1 1 . 1 0 0 . 1 1 1 1 1 1 1 1 1 1 1 . 1 0
IND-002 . . 1 . . 1 . 1 1 0 . . 1 0 0 1 2 2 2 . 2 1 1 1 1 2 2 . . .
IND-004 . . 1 . . 1 1 1 1 1 1 . 1 0 0 . 1 1 1 1 1 1 1 0 0 0 0 . 0 1
IND-083 . . 1 . . 1 . 1 0 1 . . 0 0 0 . . 1 1 1 1 1 1 1 1 1 1 . 1 2
```

```
IND-093 . . 1 . . 0 1 1 0 0 0 . 1 0 0 1 1 1 1 0 1 2 2 0 0 0 0 . 1 1
IND-051 . . 1 . . 1 1 1 0 1 1 . 1 0 0 1 1 1 2 1 2 2 2 1 1 1 1 . 2 1
-stop individuals markers
-start traits
RawHY_1  1.5000 1.5000 1.8000 1.8000 2.0000 2.0000 2.0000 2.0000
2.0000 2.0000 2.0000 2.2000 2.3000 2.5000 2.5000 2.5000
2.5000 2.8000 3.0000 3.0000 3.0000 4.1000 4.2000 4.2000
4.5000 4.5000 4.5000 4.7000 5.0000 5.0000 5.0000 5.0000
5.1000 5.2000 5.2000 5.2000 5.5000 5.5000 5.6000 5.8000
6.0000 6.0000 6.0000 7.0000
-stop traits
#endcross
-quit
-end
```

HIGH THROUGH-PUT (HTP) DNA EXTRACTION PROTOCOL

(Revised 1/5/2004)

Original Source: Michaels, S.D. and Amasino, R.M. (2001) High Throughput Isolation of DNA and RNA in 96-Well Format Using a Paint Shaker. Plant Molecular Biology Reporter **19**: 227-233.

**Note: This protocol has been modified from that described in the above paper for Arabidopsis and the equipment available in the Norman Borlaug Center.

PROCEDURE:

1. Collect Tissue for DNA extraction and place into 96-Well COSTAR plates
    a. 1 small seedling per well
    b. 2 disks cut out of mature leaves using a disposable drinking straw
    c. 1 callus

2. Add 500 ul of Extraction Buffer per tube
3. Add 1 autoclaved, stainless steel bar per tube.
4. Cap tightly with COSTAR strip caps designed for tubes. DO NOT USE ANY OTHER CAPS OR YOU WILL HAVE LEAKAGE AND POSSIBLE CROSS CONTAMINATION!!
5. Secure plates in GENOGRINDER in LPGT (DO NOT USE LIDS ON BOXES! PLEXIGLASS SPACERS SHOULD BE ADDED UNTIL THERE IS A TIGHT FIT AND EQUAL PRESSURE ACROSS ALL THE CAPS OF THE WELLS!)
6. Grind tissue at 1500 strokes per min for 2 min. (Note: Ground up tissue will be 'foamy' from SDS in extraction buffer. A brief spin to remove the 'foam' or addition of an antifoaming agent to the extraction buffer might be of benefit)
7. Place 800 ul melt-blown polypropylene filter plate on top of 1 ml deep well plate.
8. Pipet 400 ul of ground tissue and solution into wells of filter plate (wide orifice tips make this procedure easier) Note: Some extract might begin to drip before spinning plates in centrifuge. Care should be taken not to jar plates since filter plate does not securely fit over receiving plate.
9. Spin filter plate and receiving plate in large Speed Vac in LPGT without heat or vacuum until all liquid has moved through the filter plate into the receiving plate.
10. Add 400 ul IPA to all wells in 1 ml deep well plate and mix by pipetting to precipitate DNA.
11. Cap plate with sealing mat and incubate at room temperature for 10 min. Note: DO NOT INVERT PLATE TO MIX!! CAPS DO NOT FORM TIGHT ENOUGH SEAL TO PREVENT LEAKAGE!
12. Spin plates at max speed (3250 rpm) for 30 minutes to pellet DNA
13. Remove sealing mat and invert plates to remove supernatant

14. Dry pellets at room temp, using small speed-vac with rotor removed, or large centrifugal speed vac.
15. Resuspend DNA pellet in either 50 ul or 100 ul of 0.1 X TE depending on starting tissue and pipetting volume desired for PCR. (Note: I use 100 ul because it is easier to pipet 2 ul using a multi-channel pipet than 1 ul for setting up downstream PCR)
16. Check DNA isolation on 1 % agarose gel (Note: DNA extracted from callus culture will need RNAse treatment to remove abundant RNA)
17. Store DNA at -20 C in 1 ml deep well plates sealed with mats or transfer to 0.5 ml plates to take up less room (seal 0.5 ml plates with tape or sealing film. 1 ml cap mat will not fit 0.5 ml plate.)

Supplies required for each 96-well DNA extraction:

1, COSTAR Macrotube plate (COSTAR Cat # 4413)
1, Set of COSTAR strip cap tubes for COSTAR Macrotube Plate (COSTART Cat # 4418)
1, 800 ul, 0.45 um, 96-well melt blown polypropylene filter plate (Phenix Cat # F-20005)
1, 1 ml, round bottom, 96-well PP plate (Phenix Cat # M-0353)
Sealing Mat for 1.0 ml, 96-well Deep well PP plate (Phenix Cat# M-0355)
Stainless steel pins 1/8" X 1/2" (Small Parts, Inc. Cat#DWX-02-08-C, www.smallparts.com)

Chemicals Needed:

Plant DNA Extraction buffer (200 mM Tris pH 7.5, 250 mM NaCl, 25 mM EDTA pH 8.0, 05.% SDS)

Isopropyl alcohol

0.1 X TE (1 mM Tris pH 8.0, 0.1 mM EDTA pH 8.0)

APPENDIX B

SUPPLEMENTARY MATERIAL FOR CHAPTER IV

DEVELOPED MARKER DATA

Table B-1. SSLP and CAPs markers developed for mapping *ted2-1D*.

Marker	CH	POS	Primers (3' -> 5')	Col-0	La-er	Enzyme	
F9H16	I	28.00 cM	F-TGG TGA GAT ACT GAG ATT ATC CTT G	161 bp	133 bp-		
			R-GAT TCT ATT TTG CTT GGC GTA TGT G				
F8K7	I	30.00 cM	F-TTG CAG CTT CAC GGA CAA CA	102 bp	102 bp	Hae II	Col-0 cuts
			R-TGC GCT TAC TCT CTC ACT TTT G				
F12K8	I	30.20 cM	F-CGC CTC TGC TCT CTA TAT AGT GG	171 bp	148 bp-		
			R-GCC CCT GTT GTT CAA TCA AAT C				
F19G10	I	33.09 cM	F-AAC TTA ATT TCT CTC ACC CGA AG	117 bp	101 bp-		
			R-CTC CGA CAC AAC AGA GAT TCC				
T26J12.1	I	33.12 cM	F-GAC AAT TCT TAG TAA AAT AGA TTC AC	141 bp	119 bp-		
			R-CGA GCA TAT GTC TTG CTT CC				
T26J12.2	I	33.14 cM	F-CCA TTA TAC GTA TAG GCG TTC	79 bp	73 bp-		
			R-GTT TGT TCC AAG GTA AAT TAG TG				
F26F24.4	I	33.85 cM	F-CCA AGG ACC AAT GAA AGA CGC	88 bp	80 bp-		
			R-CAT CTA CAC ACA AGA AAG CAT CAA TC				
F26F24.6	I	33.94 cM	F-GGG GAA ATT AAG TTA AAG AAA GGA G	114 bp	88 bp-		
			R-TGA TCT TTG CCA ATA ATA AGA AGG C				
F5O8.1	I	34.12 cM	F-CAG CCT TGC AAA AGC GCA CC	87 bp	78 bp-		
			R-CCA AAT AAG CAG CCA TTT GTA AGG				
T23E23.5	I	34.47 cM	F-CTT CCA AAT CGT CAC TCT CTG C	100 bp	92 bp-		
			R-CAT GAG TAG ATA TCT AAC CTT AAT C				
F3I6.3	I	34.81 cM	F-GAA AAC TAA TGG AGG ATA TGT TCA AG	125 bp	101 bp-		
			R-CTA AAA ATA TCC CTA AAA ATA TGT ACA TTG				
F21J9	I	35.16 cM	F-GAG ACG AAG AAG ATG GAT TCT G	190 bp	180 bp-		
			R-CAG ATT TCT TGC CAA GTT TCA TC				
F28B23	I	36.67 cM	F-CAC ACA CTT CGA GTT CCT TG	96 bp	86 bp-		
			R-ACC CTA ATT GTG TAT CTG TAG C				
CIW12	I	39.00 cM	F-AGG TTT TAT TGC TTT TCA CA	128 bp	115 bp-		
			R-CTT TCA AAA GCA CAT CAC A				
F13K9	I	39.60 cM	F-TCT CCA CTG AAG GGG TTG AG	83 bp	56 bp-		
			R-ATG CGC GCT AGG GGA AAT TC				
nga392	I	41.64 cM	F-GGT GTT AAA TGC GGT GTT C	170 bp	162 bp-		
			R-TTG AAT AAT TTG TAG CCA TG				
nga248	I	42.17 cM	F-TCT GTA TCT CGG TGA ATT CTC C	143 bp	129 bp-		
			R-TAC CGA ACC AAA ACA CAA AGG				
nga280	I	83.83 cM	F-GGC TCC ATA AAA AGT GCA CC	105 bp	85 bp-		
			R-CTG ATC TCA CGG ACA ATA GTG C				
nga111	I	115.50 cM	F-TGT TTT TTA GGA CAA ATG GCG	128 bp	162 bp-		
			R-CTC CAG TTG GAA GCT AAA GGG				

F_2(TAM754)A MAPPING DATA

Mapping Data for F_2(TAM754)A Mapping Population

The following data is in the format used for QTL Cartographer:

```
#FileID  F2(TAM754)A TED2
#bychromosome
-type  position
-function  2
-Units  cM
-chromosomes  1
-maximum 18
-named yes
-start
-Chromosome  CHR_I  18
F9H16  29.9500
F12K8  32.1100
F19G10  33.0900
T26J12_1  33.1200
T26J12_2  33.1400
F26F24_4  33.8500
F26F24_6  33.9400
F508_1  34.1200
T23E23_5  34.4700
F3I6_3  34.8100
F21J9  35.1600
F28B23  36.6700
CIW12  39.0000
F13K9  39.6000
nga392  41.6400
nga248  42.1700
nga280  83.8300
nga111  115.5000
-stop
#endchromosome
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#bycross
-Samplesize  92
-Cross  SF2
-traits  1
-missingtrait  .
-case yes
-TranslationTable
AA  2  2
Aa  1  1
aa  0  0
A-  12 12
a-  10 10
--  -1 .
-start individuals markers
ID-004 . 1 1 . . . . . . . 1 1 1 1 1 1 2 1
ID-278 0 0 0 . . . . . . . 0 0 0 0 0 0 0 0
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ID-220 0 0 0 . . . . . 0 0 0 0 0 0 . 1
ID-360 0 0 0 . . . . . 0 0 1 1 1 1 . 2
ID-059 0 0 0 . . . . . 0 0 0 0 0 0 1 2
ID-107 0 0 0 . . . . . 0 0 0 1 1 1 1 1
ID-403 0 0 0 . . . . . 0 0 0 0 0 0 1 0
ID-461 0 0 0 . . . . . 0 0 0 0 0 0 1 1
ID-462 0 0 0 . . . . . 0 0 0 0 0 0 1 1
ID-157 0 0 0 . . . . . 0 0 0 0 0 0 0 1
ID-183 0 0 0 . . . . . 0 0 0 0 0 0 0 1
ID-209 0 0 0 . . . . . 0 0 0 0 0 0 1 1
ID-270 . 0 0 . . . . . 0 0 0 0 0 0 1 1
ID-432 0 0 0 . . . . . 0 0 0 0 0 0 0 0
ID-158 0 0 0 . . . . . 0 0 0 0 0 0 1 1
ID-021 0 0 0 . . . . . 0 0 0 0 0 0 1 1
ID-056 0 0 0 . . . . . 0 0 0 0 0 0 1 2
ID-327 0 0 0 . . . . . 0 0 0 0 0 0 1 1
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ID-055 0 0 0 . . . . . 0 0 0 0 0 0 2 2
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ID-297 2 2 2 . . . . . 2 2 2 2 2 2 2 2
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ID-223 2 2 2 . . . . . 2 2 2 2 2 2 1 1
ID-298 2 0 0 . . . . . 0 0 0 0 0 0 0 0
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ID-385 2 2 2 . . . . . 2 2 2 2 2 2 2 1
ID-024 2 2 2 1 1 1 . 1 1 . 1 1 1 1 1 1 . 0
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ID-008 2 2 2 2 2 2 2 2 2 1 1 1 1 1 1 . . 2
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ID-052 2 2 2 . . . . . . . . 2 2 1 1 1 1 0 2
ID-169 2 2 2 . . . . . . . . 2 2 2 2 2 2 2 2
ID-264 1 1 1 . . . . . . . . 1 1 1 1 1 1 1 1
ID-266 1 1 1 . . . . . . . . 1 1 1 1 0 0 2 2
ID-321 1 1 1 . . . . . . . . 1 1 1 1 1 1 1 1
ID-231 1 1 1 . . . . . . . . 1 1 1 1 1 1 1 1
ID-304 1 1 1 . . . . . . . . 1 1 1 0 0 0 0 0
ID-029 1 1 1 . . . . . . . . 1 1 1 1 1 1 2 2
ID-269 1 1 1 . . . . . . . . 1 1 1 1 1 1 1 1
ID-323 1 1 1 . . . . . . . . 1 1 1 1 1 1 1 1
ID-378 1 1 1 . . . . . . . . 1 2 2 2 2 2 0 0
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ID-417 1 1 1 . . . . . . . . 1 1 1 1 1 1 1 2
ID-222 1 1 1 . . . . . . . . 1 1 1 1 1 1 1 0
ID-305 1 1 1 . . . . . . . . 1 1 1 1 1 1 2 2
ID-199 1 1 1 . . . . . . . . 1 1 1 1 1 1 0 0
ID-258 1 1 1 . . . . . . . . 1 1 1 1 1 1 1 1
ID-460 2 2 2 . . . . . . . . 2 2 2 2 2 2 2 2
ID-429 0 1 1 . . . . . . . . 1 1 1 1 1 1 2 0
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ID-061 1 1 1 . . . . . . . . 1 1 1 1 1 1 0 1
ID-198 1 1 1 . . . . . . . . 1 1 1 1 1 1 1 2
ID-216 1 1 1 . . . . . . . . 1 1 1 1 1 1 1 0
ID-251 1 1 1 . . . . . . . . 1 1 1 1 1 1 0 0
ID-356 1 1 1 . . . . . . . . 1 1 1 1 1 1 2 2
ID-208 1 1 1 . . . . . . . . 1 1 1 1 1 1 2 2
ID-050 0 . 1 . . . . . . . . 1 1 1 1 1 1 1 2
ID-068 1 1 1 . . . . . . . . 1 1 1 1 1 1 1 1
ID-133 1 1 1 . . . . . . . . 1 1 0 0 0 0 1 1
ID-135 1 1 1 . . . . . . . . 1 1 1 1 1 1 1 1
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ID-340 1 1 1 . . . . . . . . 1 1 1 1 1 1 1 1
ID-123 1 1 1 . . . . . . . . 1 1 1 1 1 . 0 0
-stop individuals markers
-start traits
RawHY_1  7.0000 2.0000 2.2000 2.5000 3.0000 3.0000 3.0000 3.0000
3.4000 3.5000 3.5000 3.5000 3.5000 3.5000 3.6000 3.8000
3.8000 3.8000 3.8000 4.0000 4.2000 4.2000 4.2000 4.5000
5.0000 5.0000 5.0000 5.0000 5.5000 5.5000 5.6000 6.0000
6.0000 6.0000 6.2000 6.2000 6.4000 6.5000 6.5000 7.5000
7.5000 7.6000 7.8000 8.2000 8.2000 8.4000 8.5000 8.5000
8.5000 9.0000 9.2000 9.4000 9.5000 9.5000 9.8000 9.8000
10.0000 10.5000 11.0000 11.0000 11.0000 11.0000 11.0000 11.0000
11.5000 11.5000 12.0000 12.0000 12.0000 12.0000 12.2000 12.4000
12.5000 12.6000 13.0000 13.0000 13.0000 13.2000 13.4000 13.5000
13.5000 13.5000 13.5000 13.5000 14.0000 14.5000 14.5000 15.0000
15.0000 15.0000 15.4000 15.5000
-stop traits
#endcross
-quit
-end
```

VITA

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